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(54) Title: METHODS AND COMPOSITIONS FOR POTENTIATING ANTIBIOTIC ACTION AGAINST PERSISTENT/TOLERANT PATHOGENIC MICROORGANISMS

(57) Abstract: The invention provides methods and reagents for the treatment of a microbial infection or decontamination of a microbial surface contamination, particularly those characterized by a population of persistent or tolerant cells such as occur in biofilm and certain planktonic bacterial populations. The invention is further directed to methods of identifying microbial tolerance gene targets and antagonsits of these microbial tolerance gene targets as well as for identifying microbial intolerance gene targets and agonists of these microbial intolerance gene targets.

Methods and Compositions for Potentiating Antibiotic Action Against Persistent/Tolerant Pathogenic Microorganisms

Cross Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/228,042, filed 25 August 2000, the contents of which are specifically incorporated herein.

Government Support

This invention was made with government support under GM54412 and RO1 GM61162, awarded by the National Institutes of Health. The government has certain rights in the invention.

1. Background of the Invention

Many problematic infections and persistent surface contaminations are caused by slow-growing microbial cells that are very resistant to killing by antimicrobials. In some instances, these slow-growing "persistent" or "tolerant" cell populations may exist as a surface "biofilm", consisting of dispersed colonies of one or more microorganisms which can attach to virtually any conceivable organic or inorganic surface. In other instances these "persistent" or "tolerant" cell populations can exist in a freely-dispersed or "planktonic" condition not attached to any surface. For example, Pseudomonas aeruginosa forms a bacterial biofilm by producing alginate when it adheres to either mucosa or non-tissue strata such as various medical devices (see Vranes (2000) J Chemother 12: 280-5). Mixed bacterial and fungal biofilms can colonize and interfere with the function of prosthetic devices such as the silicone rubber voice prosthesis used in laryngectomized patients (see e.g. van der Mei et al. (2000) J Med Microbiol 49: 713-8). Furthermore, microbial biofilms can develop on the surfaces of external instrumentation and apparatus such as poultry processing equipment (Arnold and Silvers (2000) Poult Sci 79: 1215-21) and dental-unit water systems (Walker et al. (2000) Appl Environ Microbiol 66: 3363-7) and thereby cross-contaminate other biological and non-biological surfaces. Furthermore, persistent or tolerant populations can also exist under planktonic growth conditions (see e.g. Duguid et al. (1992) J Antimicrob. Chemother. 30(6): 791-802).

Bacterial infection is a frequent problem resulting from invasive surgical, therapeutic and diagnostic procedures and surgical procedures involving implantable medical devices present particular problems when infections arise because the infectious agent can develop into biofilms. As stated above, such a biofilm growth state may protect the microbes from clearing by the

subject's immune system. These infections have proven difficult to treat with antibiotics and so removal of the device is often necessitated. Such measures are traumatic to the patient and increase overall medical costs. Biofilm infections are recalcitrant to treatment due to the very high tolerance of biofilm cells to killing by antimicrobials. The unique feature of the biofilm is the presence of high numbers of persisting cells (Brooun et al., 2000). The biofilm does not grow in the presence of antibiotics, and the challenge for anti-biofilm drug discovery is to come up with compounds specifically targeting persistent/tolerant cells. Tolerant cells are also present in "deep-seated" infections (probably similar to biofilms); and in meningitis, for example. Examples of such infections include P. aeruginosa infections in cystic fibrosis; S. aureus infections in endocarditis; S. pneumoniae meningitis infections; and numerous other microbial biofilm infections of implants and indwelling devices like catheters. Although antibiotic resistant tolerant cells frequently occur in a biofilm growth state, it is important to note that they may also exist, in certain instances, in a planktonic (unattached) growth state in certain instances.

Microorganisms existing in a biofilm, termed sessile, grow in a protected environment that insulates them from attack from antimicrobial agents. These sessile communities can give rise to nonsessile individuals, termed planktonic, which rapidly multiply and disperse. These planktonic organisms are responsible for invasive and disseminated infections. They are the targets of antimicrobial therapy. Conventional treatments fail to eradicate the sessile communities rooted in the biofilm. Biofilms are understood to be a frequently occurring reservoir for infectious agents. The biology of biofilms is described in more detail in "Bacterial biofilms: a common cause of persistent infection," J. Costerson, P. Stewart, E. Greenberg, Science 284: 1318-1322 (1999), the contents of which are incorporated herein by reference.

Biofilms develop preferentially on inert surfaces or on non-living tissue, and occur commonly on medical devices and devascularized or dead tissues. Biofilms have been identified on sequestra of dead bone and on bone grafts, from which they can incite an invasive infection called osteomyelitis that can kill even more bone. Biofilms have been also identified on living, hypovascular tissues such as native heart valves, where they are responsible for the devastating infection called endocarditis where the microorganism not only can colonize distant locations by seeding throughout the bloodstream, but also can destroy the heart valve itself. Infections involving implanted medical devices generally involve biofilms, where a sessile community provides a reservoir for an invasive infection. The presence of microorganisms in a biofilm on a medical device represents contamination of that foreign body. The elicitation by the biofilm of clinically perceptible host responses constitutes an infection.

Sessile microorganisms can also give rise to localized symptoms, releasing antigens and stimulating antibody production that activates the immune system to attack the biofilm and the area surrounding it. Antibodies and host immune defenses are ineffective in killing the organisms in the biofilm, even though these organisms have elicited the antibody and related immune response. The cytotoxic products of the host's immunologically activated cells can be directed towards the host's own tissues. This phenomenon is seen in the mouth, where the host's response to the dental biofilm can inflame tissues surrounding the teeth and give rise to periodontitis. This phenomenon can also give rise to both local inflammation around implanted medical devices and bone resorption with resulting loosening of orthopedic and dental implants.

While host defenses may hold invasive infections in check by controlling the proliferation of planktonic organisms, this favorable equilibrium presupposes an intact immune system. Many patients in a hospital setting have compromised immune systems, rendering them more vulnerable to invasive infections once a biofilm community has become established. Patients requiring implantable medical devices may likewise have compromised immune systems, whether on a short-term or long-term basis. A poorly functioning immune system puts the host at greater risk for initial formation of a contaminated biofilm around a medical device and for the invasion of planktonic organisms into the surrounding tissues and the system. Once the planktonic organisms mount a full-scale infection, the immunocompromised host will be less likely to contain and control it, with potentially lethal results. Even in individuals not immunocompromised, microorganisms existing in a biofilm maintain a form of protection from antibiotic treatment and host defenses, and so the microorganisms in a biofilm typically cause recurrent infections and low-grade local symptoms. The biofilm, once established, can only be eradicated surgically. When a foreign object becomes contaminated with microorganisms, the only way to eliminate local and systemic infection may be to remove the contaminated foreign article. If the material being removed is essential for health, a similar article may need to be replaced in the same location; the replacement article will be especially prone to infection because of the residual microorganisms in the area.

Biofilm formation also has important public health implications for the entire otherwise healthy uninfected population. Drinking water systems are known to harbor biofilms, even though these environments often contain disinfectants. Any system providing an interface between a surface and a fluid has the potential for biofilm development. Water cooling towers for air conditioners are well-known to pose public health risks from biofilm formation, as episodic outbreaks of infections like Legionnaires' Disease attest. Turbulent fluid flow over the surface does not provide protection- biofilms can form in conduits where flowing water or other

fluids pass, with the effects of altering flow characteristics and passing planktonic organisms downstream. Industrial fluid processing operations have experienced mechanical blockages, impedance of heat transfer processes, and biodeterioration of fluid-based industrial products, all attributable to biofilms. Biofilms have been identified in flow conduits like hemodialysis tubing, and in water distribution conduits. Biofilms have also been identified to cause biofouling in selected municipal water storage tanks, private wells and drip irrigation systems, unaffected by treatments with up to 200 ppm chlorine.

Biofilms are also a constant problem in food processing environments. Food processing involves fluids, solid material and their combination. As an example, milk processing facilities provide fluid conduits and areas of fluid residence on surfaces. Cleansing milking and milk processing equipment presently utilizes interactions of mechanical, thermal and chemical processes in an air-injected clean-in-place methods. Additionally, the milk product itself is treated with pasteurization. In cheese producing, biofilms can lead to the production of calcium lactate crystals in Cheddar cheese. Meat processing and packing facilities are in like manner susceptible to biofilm formation. Non-metallic and metallic surfaces can be affected. Biofilms in meat processing facilities have been detected on rubber "fingers," plastic curtains, conveyor belt material, evisceration equipment and stainless steel surfaces. Controlling biofilms and microorganism contamination in food processing is hampered by the additional need that the agent used not affect the taste, texture or aesthetics of the product.

Therefore biofilm formation with health implications can involve those surfaces in all health-related environments, including surfaces found in medical environments and those surfaces in industrial or residential environments that are involved in those functions essential to well-being like nutrition, sanitation and the prevention of disease. Surfaces in contact with liquids are particularly prone to biofilm formation. As an example, those reservoirs and tubes used for delivering humidified oxygen to patients can bear biofilms inhabited by infectious agents. Dental unit waterlines similarly can bear biofilms on their surfaces, providing a reservoir for continuing contamination of the system of flowing and aerosolized water used in dentistry. Sprays, aerosols and nebulizers are also highly effective in disseminating biofilm fragments to a potential host or to another environmental site. It is understood to be especially important to health to prevent biofilm formation on those surfaces from whence biofilm fragments can be carried away by sprays, aerosols or nebulizers contacting the surface. Other surfaces related to health include the inner and outer aspects of those articles involved in water purification, water storage and water delivery, and those articles involved in food processing. Surfaces related to health can also include the inner and outer aspects of those household articles involved in

providing for nutrition, sanitation or disease prevention. Examples can include food processing equipment for home use, materials for infant care, tampons and toilet bowls.

Because biofilms adversely affect the ability to treat many bacterial infections as well as systems essential to public health such as water supplies and food production facilities, a number of technologies have been proposed to treat surfaces with organic or inorganic materials in an attempt to interfere with biofilm development. For example, various methods have been employed to coat the surfaces of medical devices with antibiotics (See e.g. U.S. Patent Nos. 4,107,121, 4,442,133, 4,895,566, 4,917,686, 5,013,306, 4,952,419, 5,853,745 and 5,902,283) and other bacteriostatic compounds (See e.g U.S. Patent Nos. 4,605,564, 4,886,505, 5,019,096, 5,295,979, 5,328,954, 5,681,575, 5,753,251, 5,770,255, and 5,877,243), the contents of which are specifically incorporated herein. Despite these technologies, contamination of medical devices and invasive infection therefrom continues to be a problem. Accordingly, it would be useful to have new methods and compositions for treating persistent/tolerant microorganismal infections which exist as biofilms in a sessile growth state or, in certain instances, as freely circulating planktonic cell populations. These same treatment strategies may also be applied to decontamination strategies for environmentally-exposed organic and inorganic surfaces that are susceptible to biofilm accumulation.

The present invention relates to methods and compositions for affecting the survival of microbial "persistent" or "tolerant" cell populations, particularly, but not exclusively, those existing as a microbial "biofilm". The invention relates to the targeting of "microbial tolerance genes" as well as "microbial intolerance genes" which are involved in regulating programmed cell death pathways in such populations.

2. Summary of the Invention

The invention provides methods and compositions for the antimicrobial targeting of tolerance /persistence pathways in infectious microbes. These tolerance/persistence pathways act, in part, to inhibit microbial programmed cell death in general and autolysis pathways in particular. The invention relates to the observation that mutations in microbial programmed cell death pathway genes affect the survival of infectious microbes in the presence of antimicrobials. The invention provides methods and reagents for targeting microbial tolerance genes in general and microbial programmed cell death pathway genes in particular in order to potentiate killing of "persistent" or "tolerant" cell populations by antimicrobial agents, such as bacterial antibiotics. The invention takes advantage of particular components that make up the signal transduction pathway for programmed cell death. Such components are optimal targets of drugs that will activate microbial programmed cell death and thereby aid in the elimination of pathogens by

antibiotics. The invention is particularly well adapted to the treatment of microbial infections and surface contaminations existing as slow-growing populations of cells such as in biofilms. Such biofilm infections are characterized by tolerance to and persistence against currently available antibiotic treatments.

In certain embodiments, the invention provides methods and reagents for inhibiting the survival of a microbe existing in as an internal infection or an external surface contamination. In preferred embodiments, the invention provides methods and reagents for the treatment or decontamination of a microbial biofilm. In one particular application, the invention provides a method of inhibiting the survival of a microbial population by contacting the microbial biofilm with a first composition, which includes an inhibitor of microbial tolerance, and with a second composition, which includes an antibiotic agent, in order to inhibit the survival of the microbe. The inhibitor of microbial tolerance may be an inhibitor of a microbial tolerance gene, or may be an activator of programmed cell death gene such as an autolysis-promoting gene. As used herein, genes which promote programmed cell death, and whose activity, when activated by an agonist, promotes programmed cell death, are referred to as "microbial intolerance gene" targets. Preferred "microbial intolerance gene" targets include the "autolysis genes" encoding autolysins, such as LytA in *S. pneurmoniae* and LytM in *S. aureus*; AmiB, MltB, and SLT in *E.coli* as well as their homologs in *P. aeruginosa*, as well as other bacterial autolytic genes known in the art. The invention further provides certain combination therapies using, for example, an inhibitor of a microbial tolerance gene or an activator of a microbial intolerance gene - e.g. a programmed cell death pathway gene- in combination with a second composition which includes an antibiotic agent, thereby inhibiting the survival of persistent/ tolerant microbial cells, such as those in certain biofilm infections.

In preferred embodiments, the tolerance gene targeted for inhibition is SarA. In other embodiments, the tagret gene is Lon or RelA. In other preferred embodiments, the intolerance gene targeted for activation is LytA, LytM, AmiB, MltB, or SLT. The antibiotic agent optionally used in the subject invention may be any known antimicrobial. For example an antibiotic such as penicillin, mitomycin, ciprofoxacin, vancomycin or tobramycin. Optionally, the method of the invention provides for the administration of yet a third composition which includes an inhibitor of a second microbial tolerance gene, or an activator of a microbial intolerance gene. The targeting of two tolerance genes, optionally in combination with a conventional antimicrobial antibiotic, provides advantages in eliminating persistor cells present in the biofilm population. In addition, the same advantages may be realized by targeting a microbial intolerance gene for activation in combination with the inhibition of a single micobial tolerance

gene, along with a conventional antimicrobial antibiotic. Further still, the advantages of a combination therapeutic may be achieved by targeting two separate micobial intolerance genes for activation, preferrably in combination with a conventional antimicrobial antibiotic.

The invention further provides compositions for inhibiting the survival of a microbial biofilm. Preferred compositions include at least two compositions: a first composition which includes an inhibitor of a microbial tolerance gene, and a second composition which includes an antibiotic agent. In preferred embodiments, the microbial tolerance gene inhibitor is an antisense oligonucleotide or a polypeptide ligand antagonist and the antibiotic agent is penicillin, mitomycin, ciprofoxacin, vancomycin or tobramycin. In certain preferred embodiments, a second tolerance gene inhibitor is included which targets a second microbial tolerance gene. The targeting of two tolerance genes, optionally in combination with a conventional antimicrobial antibiotic, provides advantages in eliminating persistor cells present in the biofilm population. Other preferred compositions include at least two compositions where the first is an activator of a microbial intolerance gene and the second is an antibiotic agent. The targeting of two intolerance genes for activation or one tolerance gene for activation and one intolerance gene for activation, and optionally in combination with a conventional antimicrobial antibiotic, is also an aspect of the invention.

3. Brief Description of the Figures

Figure 1 shows that killing by ciprofloxacin of *P. aeruginosa* MexAB^{++} and MexAB^{-} cells growing as a biofilm.

Figure 2 (A) shows the time-dependent penicillin-induced killing of *S. aureus* ALC. The wild type strain is RN 6390 and the the *sarA* mutant strain is ALC 488. The concentration of penicillin was 0.4 ug/ml (10x MIC). **Figure 2 (B)** shows the dose-dependent killing of *S. aureus* with mitomycin C - the concentration of mitomycin C was varied from 0.8 ug/ml (1xMIC) to 8 ug/ml (10xMIC).

Figure 3 shows that a *lon* mutation decreases the survival while a *SulA* mutation increases the survival of *E. coli* which has been challenged with increasing concentrations of mitomycin.

Figure 4 shows the killing of *S. aureus* by heat- cells were placed in a 60°C water bath and samples were taken for plating at times indicated.

4. Detailed Description of the Invention

4.1. General

The invention relates to methods and compositions for treating microbial infections or microbial contamination, particularly infections and contaminations which exist in a biofilm-type growth state. The invention relates in part to the observation that inactivation of certain genetic pathways can potentiate cell killing by antibiotics and other toxic agents against otherwise resistant "persistor" cell populations in non-growing microbial population such as occur in so-called bacterial biofilms. The invention therefore provides a solution to the problem of eliminating these resistant persistor cells growing in biofilms. Unlike planktonic cells, biofilm cells are protected from the immune system by a polysaccaride matrix which shields rare antibiotic resistant "persistor" cells from scavanging by the immune system. These remaining persistor cells are responsible for biofilm regrowth when the antibiotic concentration drops or when the treatment is discontinued based on the apparent absence of infection. The invention provides for the treatment of biofilm infections or the decontamination of biofilm-contaminated surfaces by contact with at least one persistor or "tolerance" gene antagonist compound- alone or in combination with a conventional antimicrobial or antibiotic agent.

4.2. Definitions

The term "bioavailable" is meant to refer to an appropriate location, orientation or formulation of a compound for performance of the compound's bioactivity.

"Biofilm" refers to an accumulation of organisms on a surface. A mature biofilm can comprise a colony of microorganisms resident upon a surface surrounded by an exopolysaccharide.

The phrase "effective amount" refers to an amount of the disclosed antifouling compounds that significantly reduces the number of organisms that attach to a defined surface (cells/mm2) relative to the number that attach to an untreated surface. Particularly preferred are amounts that reduce the number of organisms that attach to the surface by a factor of at least 2. Even more preferred are amounts that reduce the surface attachment of organisms by a factor of 4, more preferably by a factor of 6. An effective amount of the disclosed antifouling compound is said to inhibit the formation of biofilms, and to inhibit the growth of organisms on a defined surface. The term "inhibit," as applied to the effect of an antifouling compound on a surface includes any action that significantly reduces the number of organisms that attach thereto.

The terms "infectious microorganisms" or "infectious agents" as used herein refers to disease causing or contributing bacteria (including gram-negative and gram-positive organisms, such as Staphylococci sps. (e.g. Staphylococcus aureus, Staphylococcus epidermis), Enterococcus sp. (E. faecalis), Pseudomonas sp. (P. aeruginosa), Escherichia sp. (E. coli), Proteus sp. (P. mirabilis)), and fungi (including Candida albicans).

The term "microbial tolerance gene" refers to bacterial genes which function to inhibit one or more funtions of a bacterial cell death pathway. Accordingly, "microbial tolerance genes" of the invention include known components of bacterial cell death pathways, as described herein and as known in the art, as well as such additional bacterial cell death-inhibiting genes which may be identified using the methods and compositions described herein.

The term "microbial intolerance gene" refers to bacterial genes which function to activate or promote one or more funtions of a bacterial cell death pathway. Accordingly, "microbial intolerance genes" of the invention include known components of bacterial cell death pathways, as described herein and as known in the art, as well as such additional bacterial cell death-inhibiting genes which may be identified using the methods and compositions described herein.

The term "microbe" as used herein is meant to include any prokaryotic organism, such as a bacterium, or primitive eukaryotic organism, such as a fungus, which is capable of infecting a human or mammalian organism. As used herein, the term "microbe" is meant to particularly include those organisms capable of existing as stable populations on a contaminated solid substrate. As used herein, the term "microbe" would include a bacterium selected from the group comprising: Helicobacter pyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium kansaii, Mycobacterium gordonae, Mycobacterium leprae, Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus faecalis, Streptococcus bovis, Streptococcus anginosus, Streptococcus pneumoniae, pathogenic Campylobacter species, pathogenic Enterococcus species, Haemophilus influenzae, Bacillus antracis, Corynebacterium diphtheriae, Erysipelothrix rhusiopathiae, Clostridium perfringers, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasturella multocida, pathogenic Bacteroides fragilis group species , Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira, and Actinomyces isrealli. The term "microbe" is further meant to include a fungus selected from the group comprising: Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatidis, Chlamydia trachomatis, and Candida albicans.

A "pharmaceutically effective amount" refers to an appropriate amount to obtain a therapeutic effect. Toxicity and therapeutic efficacy of compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining The Ld50 (The Dose Lethal To 50% Of The Population) And The Ed50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. The effective amount may vary within a range depending upon the dosage form employed and the route of administration utilized. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture.

"Pharmaceutical effective carrier" refers to a physiologically acceptable carriers or excipient. Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by, for example, injection, inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration. For therapy, the compounds of the invention can be formulated for a variety of loads of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remmington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the compounds of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included. For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles

(e.g., ationd oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated to give controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner. For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch. The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.. The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides. In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. in addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally known in the art. A wash solution can be used locally to treat an injury or inflammation to accelerate healing.

The term "surface", as used herein, refers to any surface whether in an industrial or a medical setting, that provides an interface between an object and a fluid, permitting at least intermittent contact between the object and the fluid. A surface, as understood herein, further provides a plane whose mechanical structure, without further treatment, is compatible with the adherence of microorganisms. Surfaces compatible with biofilm formation may be smooth or irregular. Fluids contacting the surfaces can be stagnant or flowing, and can flow intermittently or continuously, with laminar or turbulent or mixed rheologies. A surface upon which a biofilm forms can be dry at times with sporadic fluid contact, or can have any degree of fluid exposure including total immersion. Fluid contact with the surface can take place via aerosols or other means for air-borne fluid transmission.

As used herein, the terminology "surfaces found in medical environments" includes the inner and outer aspects of various instruments and devices, whether disposable or intended for repeated uses. Examples include the entire spectrum of articles adapted for medical use, including scalpels, needles, scissors and other devices used in invasive surgical, therapeutic or diagnostic procedures; implantable medical devices, including artificial blood vessels, catheters and other devices for the removal or delivery of fluids to patients, artificial hearts, artificial kidneys, orthopedic pins, plates and implants; catheters and other tubes (including urological and biliary tubes, endotracheal tubes, peripherably insertable central venous catheters, dialysis catheters, long term tunneled central venous catheters, peripheral venous catheters, short term central venous catheters, arterial catheters, pulmonary catheters, Swan-Ganz catheters, urinary catheters, peritoneal catheters), urinary devices (including long term urinary devices, tissue bonding urinary devices, artificial urinary sphincters, urinary dilators), shunts (including ventricular or arterio-venous shunts); prostheses (including breast implants, penile prostheses, vascular grafting prostheses, heart valves, artificial joints, artificial larynxes, otological implants), vascular catheter ports, wound drain tubes, hydrocephalus shunts, pacemakers and implantable defibrillators, and the like. Other examples will be readily apparent to practitioners in these arts. Surfaces found in the medical environment also include the inner and outer aspects of pieces of medical equipment, medical gear worn or carried by personnel in the health care setting. Such surfaces can include counter tops and fixtures in areas used for medical procedures or for preparing medical apparatus, tubes and canisters used in respiratory treatments, including the administration of oxygen, of solubilized drugs in nebulizers and of anesthetic agents. Also included are those surfaces intended as biological barriers to infectious organisms in medical settings, such as gloves, aprons and faceshields. Commonly used materials for biological barriers may be latex-based or non-latex based. Vinyl is commonly used as a material for non-latex surgical gloves. Other such surfaces can include handles and cables for medical or

dental equipment not intended to be sterile. Additionally, such surfaces can include those non-sterile external surfaces of tubes and other apparatus found in areas where blood or body fluids or other hazardous biomaterials are commonly encountered.

4.3. Antimicrobials

In preferred embodiments, the invention provides for the administration of an antimicrobial agent, such as a know antibiotic, in conjunction with an inhibitor or repressor of a microbial tolerance or persistor gene. Preferably, the antimicrobial is a bacterial antibiotic such as penicillin, mitomycin, ciprofoxacin or antibiotic quinolones. Other antimicrobial antibiotics for use in the invention include: Amikacin, Aminosalicyclic acid, Amoxicillin, Amphotericin B, Ampicillin, Ampicillin, Azithromycin, Aztreonam, Bismuth subsalicylate, Carbenicillin, Cefixime, Cefmetazole, Cefotaxime, Cefoxitin, Cefotetan, Ceftazidime, Ceftazime, Ceftizoxime, Ceftrozaxime, Ceftriaxone , Cefuroxime, Cefuroxime axetil, Cephalosporin, Chloramphenicol, Ciprofloxacin, Clarithromycin, Clavulanic acid, Clindamycin, Oral Clincamycin, Clofazimine, Dapsone, Dideoxyinosine, Doxycycline, Erythromycin, Ethambutol, Ethonamide, Fluconazole, Fluoroquinolone, Fluoroquinolone, Foscarnet, Ganciclovir, Gentaminicin, Imipenem, Intraconazole, Isoniazid, Kanamycin, Ketoconazole, Methoxazole, Metronidazole, Mezlocillin, Miconazole, Minocycline1, Nitrofurantoin, Ofloxacin, Penicillin, Piperacillin/tazobactam, Quinupristin/dalfopristin, Rifampin, Sparfloxacin, Streptomycin, Sulfonamide, Sulfisoxazole, Tazobactam, Teicoplanin, Tetracycline1, Tetracycline HCI, Ticarcillin, Tobramycin, Topical clindamycin, Trimethoprim-sulfmethoxazole, Vancomycin, Vidarabine, and Zydovudine.

4.4. Microbial Tolerance Gene Targets and Intolerance Gene Targets

The invention provides "persistor" or "tolerance" genes which are to be targeted for repression by, for example a peristor or tolerance gene or protein antagonist. In preferred embodiments, the persistor or tolerance gene target is a bacterial programmed cell death gene. The invention further provides "intolerance" genes which are to be targeted for activation by, for example an "intolerance gene" agonist or an intolerance gene overexpression vector. The "tolerance gene" and "intolerance gene" targets of the pathway are described below and relate, in general, to known bacterial cell death pathway repressors and activators respectively. The invention further provides for screens for additional bacterial "tolerance" and "intolerance" gene targets using appropriate screens as described herein.

A number of genes that affect tolerance/persistence of planktonic cells in several species have been reported in the literature (effects of these genes on biofilms have not been reported). In no case was a broad panel of antimicrobials tested; it is possible that some of these genes code for factors affecting cell survival to all lethal conditions. The present invention relates to genetic

pathways affecting the survival of persistor cells existing in a biofilm population. In particular, the invention in part is based upon the finding that genetic pathways affecting bacterial programmed cell death are involved in the antimicrobial resistance of persistor cell subpopulations present in biofilms.

Similarly to metazoa, Programmed Cell Death (PCD) plays an important role in a number of developmental processes in bacteria, such as lysis of the mother cell in sporulation, lysis of vegetative cells in myxobacterial fruiting body formation, and DNA transformation liberated from cells of Streptococci undergoing spontaneous autolysis. Thus it may be that microorganisms have also evolved programmed death of defective cells. Perhaps the most common observation of possible PCD in bacteria is autolysis of cells exposed to antibiotics and other harmful conditions. Autolysis is a self-digestion of the cell wall by peptidoglycan hydrolases, autolysins (Holtje, J. V. (1995) Arch Microbiol. 164:243-254; Shockman, et al. (1996) Microb Drug Resist 2:95-98). Both synthesis and hydrolysis of peptidoglycan are necessary for building the cell wall, and at least some autolysins are part of this normal cell growth activity. Traditionally, autolysis has been viewed as a maladaptive disbalance in these processes caused by inhibition of cell growth. We will review data suggesting that autolysis represents PCD in bacteria. Other instances of bacterial cell death, not necessarily tied to autolysis, also point to a PCD process and will be considered in this review. The cases we will consider are primarily those where particular genes affecting cell survival have been identified.

It is not immediately apparent that a defective unicellular organism will benefit from committing suicide. This simple consideration has no doubt impeded a serious inquiry into programmed death in bacteria. At the same time, it is becoming increasingly apparent that bacteria live and die in complex communities that in many ways resemble a multicellular organism. Release of pheromones induces bacteria in a population to respond in concert by changing patterns of gene expression, "quorum sensing" (Fuqua, et al. (1998) Curr Opin Microbiol 1:183-189; Hardman, et al. (1998) Antonie Van Leeuwenhoek 74:199-21038; Pesci, et al. (1997) Trends Microbiol 5:132-134; discussion 134-135); reactive oxygen species cause self-aggregation in E. coli (Budrene, et al. (1991) Nature 349:630-633) a behavior thought to provide mutual protection to the cells. Most bacterial species actually do not live as planktonic suspensions in vivo, but form complex biofilms, tightly knit communities of cells (Costerton, (1999) Science 284:1318-132219). From this perspective, programmed death of damaged cells may be beneficial to a multicellular bacterial community. For example, suicide could limit the spread of a viral infection. In the case of serious damage by toxic factors, cells will donate their nutrients to their neighbors, instead of draining resources from their kin in a futile attempt to

repair. Finally, elimination of cells with damaged DNA would contribute to maintaining a low mutation rate.

A number of observations suggest that programmed cell death occurs in bacteria through a complex pathway involving multiple regulatory genes. For example, several mutations in unrelated bacteria were reported to lead to a significant increase in survival without affecting the ability of antibiotics to inhibit cell growth, and with no other visible phenotype. In *E. coli*, mutations in HipA,B regulators produce up to 1, 000 fold increase in the number of surviving cells treated with cell wall inhibitors, DNA damaging agents, high temperature (see Black, D.S. et al. (1994), J. Bacteriol. 176:4081; Black, D.S. et al. (1991), J. Bacteriol. 173:5732; Moyed, H.S. et al. (1983), J. Bacteriol. 155:768; Moyed, H.S. et al. (1986), J. Bacteriol. 166:399; Scherrer, R. et al. (1988), J. Bacteriol. 170:3321), and fluoroquinolone antibiotics (Falla, T.J. et al. (1998), Antimicrob. Agents Chemother. 42:3282; Wolfson, J.S. et al. (1990), Antimicrob. Agents Chemother. 34:1938; (Kaldalu and Lewis, unpublished). A null mutation of the SulA regulator that inhibits division of cells damaged by mutagens protects E. coli from killing by quinolones (Piddock, L.J. et al. (1992), Antimicrob. Agents Chemother. 36:819). In Streptococcus pneumoniae, a mutation in a gene vncS coding for a membrane signaling kinase protected cells from killing by cell wall inhibitors (Novak, R. et al. (1999), Nature 399:590). We suggested that the existence of regulators of cell death indicates the presence of a Programmed Cell Death (PCD) pathway in bacteria (Lewis, K. (2000), Microbiol. Mol. Biol. Rev. 64:503). Cidal antibiotics then do not kill cells; rather, defects induced by antibiotics activate PCD. This logic is identical to what we know about apoptotic death of animal cells damaged by toxins. Several cases of adaptive PCD in bacteria have been well documented and include autolysis of the mother cell during sporulation of B. subtilis (Smith, T.J. et al. (1995), J. Bacteriol. 177:3855; Nugroho, F.A. et al. (1999), J. Bacteriol. 181:6230); release of DNA through autolysis during transformation in populations of S. pneumoniae (Mortier-Barriere, I. et al. (1998), Mol. Microbiol. 27:159); and autolysis of vegetative cells during fruiting body formation in Myxococcus xanthus (Mueller, C. et al. (1991), J. Bacteriol. 173:7164; Downard, J. et al (1995), Mol. Microbiol. 16:171). The case for PCD playing a role in bacterial development and sex is strong; whether death of damaged cells is governed by PCD as well remains an intriguing open question.

Practical consequences of a bacterial PCD pathway are significant. Activating PCD may be used to potentiate the action of cidal antibiotics. In this regard, potential inhibitors of cell death are of especial interest – these could be used as targets for drug development. For example, in *S. aureus*, the SarA protein may be such an inhibitor. SarA is a global transcriptional regulator

of virulence factors (see Cheung, A.L. et al. (1992), Proc. Natl. Acad. Sci. USA 89:6462). SarA controls expression of another global virulence factor regulator agr (Heinrichs, J.H. et al. (1996), J. Bacteriol. 178:418), and also directly binds to the promoters of hla (alpha-hemolysin) (Cheung, A.L. et al. (1994), J. Bacteriol. 176:580), spa (protein A) (Cheung, A.L. et al. (1997), Immun. 65:2243), and fnbA (fibronectin binding protein A gene) (Wolz, C. et al. (2000), Mol. Microbiol. 36:230). Furthermore, the crystal structure of SarA was recently reported. Schumacher, M.A. et al. (2001) Nature 409:215.

Programmed cell death (PCD) in bacteria plays an important role in developmental processes, such as lysis of the mother cell during sporulation of Bacillus subtilis and lysis of vegetative cells in fruiting body formation of Myxococcus xanthus. The signal transduction pathway leading to autolysis of the mother cell includes the terminal sporulation sigma factor EsK which induces the synthesis of autolysins CwlC and CwlH. An activator of autolysin in this, and other PCD processes is yet to be identified. Autolysis plays a role in genetic exchange in S. pneumoniae, and the gene for the major autolysin lytA is located in the same operon with recA. DNA from lysed cells is picked up by their neighbors and is recombined into the chromosome by RecA. LytA requires an unknown activator controlled by a sensory kinase VncS. Deletion of vncS inhibits autolysis, and also decreases killing by unrelated antibiotics. This observation suggests that PCD in bacteria serves to eliminate damaged cells, similarly to apoptosis of defective cells in metazoa. The presence of genes affecting survival without changing the growth sensitivity to antibiotics (vncS; lytA; hipAB; sulA; mar) indicate that bacteria are able to control their fate. Elimination of defective cells could limit the spread of a viral infection and donate nutrients to healthy kin cells. An altruistic suicide would be challenged by the appearance of asocial mutants without PCD; and by the possibility of maladaptive total suicide in response to a uniformly present lethal factor or nutrient depletion. It is proposed that a low rate of mutations serves to decrease the probability of asocial mutants without PCD taking over the population. It is suggested that persistors, rare cells that are resistant to killing, have PCD disabled and ensure population survival. It is suggested that lack of nutrients leads to stringent response that suppresses PCD, producing a state of tolerance to antibiotics allowing cells to discriminate between nutrient deprivation and unrepairable damage. High levels of persistors are apparently responsible for the extraordinary survival properties of bacterial biofilms, and genes affecting persistence appear to be promising targets for development of drugs aimed at eradicating recalcitrant infections. PCD in unicellular eukaryotes is considered, including aging in yeast. Apoptosis-like elimination of defective cells in yeast and protozoa suggest all unicellular life forms evolved altruistic programmed death that serves a variety of useful functions. The PCD genes and other tolerance or persistance gene targets for use in the

invention are discussed in detail below in the context of the process and pathways in which they function.

Sporulation

Apoptosis in eukaryotic cells was discovered in studies of C. elegans development - death of certain cells is a necessary stage in ontogenesis (Metzstein, et al. (1998) Trends Genet 14:410-41661). Similarly, autolysis is part of the developmental process in a number of bacterial species.

In sporulating B. subtilis, the mother cell is actively lysed prior to release of the spore. Three autolysins have been identified that participate in mother cell lysis. CwlB is the major autolysin produced at the end of exponential growth phase. A double cwlB/cwlC mutant was defective in mother cell lysis (Smith, et al. (1995) J Bacteriol 177:3855-386288), but single mutants showed lysis. Similarly, double (but not single) cwlC/cwlH mutants were defective in mother cell lysis (Nugroho, et al. (1999) J Bacteriol 181:6230-623770). CwlC becomes the major autolysin at late sporulation stage. Both CwlC and a minor autolysin CwlH are transcribed by sporulation-specific EsK RNA polymerase and require GerE (a coat protein transcriptional activator) for expression (Nugroho, et al. (1999) J Bacteriol 181:6230-623770). Expression of sK in the process of sporulation has been described in much detail (Kroos, et al. (1999) Mol Microbiol 31:1285-1294; Levin, et al. (1998) Curr Opin Microbiol 1:630-63552; Stragier, et al. (1996) Annu Rev Genet 30:297-341). sK is the final regulator in a complex pathway that begins with SpoOA, a transcriptional factor that integrates signals from nutritional status and cell cycle, and in turn activates a cascade of interdependent sigma factors in the forespore and mother cell. EsK is responsible for spore coat and cortex formation, and for mother cell lysis. It might seem that the knowledge of the signal transduction pathway controlling programmed death of the mother cell is fairly complete. However, the mere presence of autolysin is not sufficient for lysis. Autolysis usually requires an additional, unidentified factor to activate an autolysin. Indeed, an autonomously active CwlB, for example, would exterminate cells at the end of the exponential state of growth. An additional activator released after the spore is formed would allow to specifically target the mother cell for autolysis.

The obvious function of mother cell autolysis is to eliminate a barrier that could interfere with the outgrowth of a germinating spore. There might be an additional function of autolysis - nutrients released by the mother cell could be used by kin cells, helping them complete the energy-demanding process of sporulation. It is interesting to note that in B. subtilis, rapid autolysis is also observed if cells are transferred into a medium lacking a carbon source. Autolysis is activated by a decrease in pmf, rather than by a drop in ATP (Jolliffe, et al. (1981)

Cell 25:753-76348). Apparently, sporulation can not proceed in the complete absence of nutrients. Altruistic lysis under such conditions might provide some of the kin cells with enough of an energy source to sporulate. A simpler explanation of course is that B. subtilis can not cope with a sudden decrease in energy, and cell lysis is a maladaptive consequence of a breakdown in the control of the cell wall synthesis machinery. While one can not discriminate between these possibilities on the basis of what we currently know, the behavior of a ruminant bacterium Fibrobacter succinogenes under similar circumstances provides a telling example suggesting bacteria do not leave much to chance. A considerable part of F. succinogenes lyse in a logarithmically growing culture, but if a sugar is withdrawn, autolysis is inhibited by a secreted protease that digests the autolysins (Wells, et al. (1996) J Dairy Sci 79:1487-1495). This behavior is the direct opposite of what transpires in a B. subtilis population under similar conditions. The ruminant symbiont converts sugar into protein and might be lysing to supply the host with valuable nutrients. When sugar is not provided, as inbetween the host meals, there is no point for the symbiont to lyse, and lysis is specifically inhibited.

M. xanthus development

Fruiting body formation and sporulation in Myxococcus bacteria is another example of controlled death aiding development (Downard, et al. (1995) Mol Microbiol16:171-175; Mueller, et al. (1991) J Bacteriol 173:7164-7175; Rosenbluh, et al. (1990) J Bacteriol 172:4307-4314). "Autocides", which are fatty acids, as well as glucosamine, induce autolysis in dense cultures of M. xanthus and are required for normal fruiting body development and sporulation. The pathway leading from these inducers to autolysis is not known, and neither is the exact role of autolysis in development. Most likely, lysis of vegetative cells is altruistic suicide, and the released nutrients feed the fruiting body and developing spores.

Programmed death and sex.

Another example of specialized adaptive autolysis related to development comes from studies of genetic exchange. In a number of species, autolysis is part of a well-controlled mechanism of natural transformation. The released DNA is picked up by cells that did not lyse. Interestingly, the gene for the main autolysin in S. pneumoniae, lytA is located in the same operon with recA which is responsible for homologous recombination with the incoming DNA (Mortier-Barriere, et al. (1998) Mol Microbiol 27:159-170). Both RecA and LytA are induced by the quorum sensing factor, a peptide pheromone that accumulates at high cell density.

S. pneumoniae lysis by antibiotics

The three types of specialized bacterial autolysis participating in development and genetic exchange described above are clearly adaptive, and are controlled by programmed death pathways. A number of intriguing observations suggest programmed death in bacteria also occurs in response to damage, analogously to apoptosis of defective animal cells.

In a recent study of S. pneumoniae, a collection of transposon insertion mutants was screened for resistance to lysis by penicillin (Novak, et al. (1998) Mol Microbiol 29:1285-1296). Mutants in over 10 genes were obtained, and two of them were identified and characterized. One mutation was in a psa locus coding for an ABC-type Mn translocase (Novak, et al. (1998) Mol Microbiol 29:1285-1296). The mutation had a pleiotropic phenotype, and its connection to lysis is unclear. Another mutant was more interesting - the affected gene named vncS was homologous to VanS, the kinase of the two-component regulatory system controlling induction of vancomycin resistance in Enterococcus faecalis (Novak, et al. (1999) Nature 399:590-593). Remarkably, vncS mutants were reported to be resistant to killing by a range of antibiotics hitting unrelated targets (the cell wall; DNA gyrase; the ribosome; it is not clear if death from antibiotics not targeting the cell wall resulted from lysis). At the same time, growth of the vncS mutant was inhibited by antibiotics as effectively as in the wild type, showing that antibiotics were able to act normally against their targets in vncS cells. In S. pneumoniae, an autolysin LytA is responsible for autolysis, and lytA mutants are resistant to killing by cell wall inhibitors like penicillin (Tomasz, et al. (1970) Nature 227:138-140); antibiotics that are not cell wall inhibitors were not tested with the lytA mutant). LytA was normally expressed in the vncS strain. Apparently, VncS does not control the synthesis of LytA, but rather regulates expression of an unknown factor that activates LytA in response to antibiotic action. vncR, a gene for a putative response regulator, was located in the same operon with vncS. A deletion of vncR had no obvious phenotype and showed normal autolysis. It was suggested that signal transduction from VncS might pass via redundant response regulators, one of which is VncR. It is not known what VncS might be sensing..

What can be concluded from these experiments is that VncS is a component of the autolytic pathway, and is required for autolysis induced by antibiotics. But why do antibiotics induce autolysis? One possibility is that the normal pathway of VncS-dependent autolysis in S. pneumoniae is somehow unnaturally activated by different antibiotics (Novak, et al. (1999) Nature 399:590-593). Alternatively, elimination of defective cells damaged by antibiotics could represent programmed death.

Persistence - survival of rare cells

One potentially severe problem with programmed death in bacteria is that suicide could eliminate all cells of a clonal population in response to damage. Indeed, a chemical toxin diffusing into a population can reach, and equally damage all cells. A resulting programmed death of the entire population would be counter-productive. However, it appears that bacteria devised an intelligent strategy to avoid such a disastrous outcome. In 1944, Bigger discovered that addition of penicillin to staphylococci produced lysis, but even after prolonged incubation, a small fraction of cells (10-6) remained fully viable (Bigger, J. W. (1944) Lancet ii:497-500). These "persistors" are not mutants by two criteria - their growth is completely sensitive to penicillin, and their progeny are not more resistant to lysis by penicillin than the original population. The existence of persistors suggests that a bacterial cell potentially has the choice of whether to live or die, and the real puzzle is not how the rare cells survive, but why the majority of cells choose to be killed by antibiotics. The phenomenon of persistence has been recorded in a wide variety of bacterial species treated with a range of deleterious factors (Koch, A. L. (1996). Similarities and differences of individual bacteria within a clone., pp. 1640-1651. In Neidhardt, et al. (Eds.) Cellular and Molecular Biology. ASM Press, Washington). For example, Luria and Laterjet described a "subpopulation" of E. coli with increased resistance to UV (Luria, et al. (1947) J. Bacteriol. 53); we have observed that a small fraction of cells in a P. aeruginosa biofilm population are virtually insensitive to killing by antibiotics (Brooun, et al. (2000) Antimicrob Agents Chemother 44:640-646). It seems that bacteria came up with an optimal strategy to respond to external damaging factors - the majority of cells have a program that will determine whether to repair damage or activate suicide, while a small fraction of "persistors" have the suicide program disabled in case the damaging factor reaches the entire population.

The nature of persistence is unknown, but it does not seem likely that a special regulatory program is able to assign as little as 10-6 cells to perform a particular function, in this case survival. In bacteria, the number of molecules of a regulator can be very low, for example about 10 molecules of LacI repressor per one E. coli. If one or more of the regulators controlling cell survival (like SulA or HipA, see below) were present in low amounts, a random variation in the number of these proteins would produce rare persisting variants. The rate of persistors would then depend on the mean number of a regulator produced, and on the variation of the mean. The evolved setting of this mean and its variation would depend on the need for making persistor cells.

Genes controlling death of E. coli

Similarly to vncS of S. pneumoniae, a number of regulatory genes were found to dramatically affect cell survival in E. coli without influencing the ability of a cell to grow in the presence of a lethal factor.

A targeted screen for E. coli genes affecting persistence was performed by Moyed and coworkers (Moyed, et al. (1983) J Bacteriol 155:768-775). The rationale was to enrich an EMS-mutagenized population with cells surviving penicillin treatment and then screen for colonies producing higher numbers of penicillin persistors. Only mutants whose growth was normally inhibited by penicillin were examined further. This straightforward approach led to the identification of three independent hip (high persistence) loci. All hip mutants produced about 1000 fold more persistent cells as compared to the wild type. One of the loci, hipAB was cloned and sequenced (Black, et al. (1991) J Bacteriol 173:5732-5739; Moyed, et al. (1986) J Bacteriol 166:399-403). This was the first report of bacterial genes specifically involved in regulation of cell death. A knockout of the wild type hipA gene did not have an apparent phenotype, while a hipB knockout was not obtained, indicating that a null mutant is non-viable. A deletion of both hipA and hipB was obtained, and this strain had similar persistence in the presence of penicillin as compared to the wild type (Black, et al. (1994) J Bacteriol 176:4081-4091). Biochemical studies have shown that HipB is a transcriptional repressor that binds to the promoter region of the hip operon. HipB is a 10 Kd HTH DNA binding protein that forms a dimer and forms a tight 1:1 complex with HipA. Moderate expression of cloned wild type HipA produced the same phenotype as the original hip(A or B) mutation - the level of cells persisting penicillin killing was increased (Falla, et al. (1998) Antimicrob Agents Chemother 42:3282-3284). The fact that a null hipB is likely lethal indicates that overexpression of HipA causes death. Indeed, high level of expression of HipA from a controllable promoter inhibited cell growth, although cell survival was not examined under these conditions (Falla, et al. (1998) Antimicrob Agents Chemother 42:3282-3284). Apparently, the hipAB locus has the potential to act both as an inhibitor of cell death and as a killing factor. It was suggested that mutants with increased persistence that were localized to either hipA or hipB loci have a decreased affinity of HipA/HipB binding, and thus have a higher (and moderate) level of free HipA that protects cells from killing by ampicillin. Most importantly, the hip mutants showed increased resistance to factors unrelated to ampicillin. Mutated cells had a 1,000 fold higher survival rate to thymine starvation that leads to DNA degradation (Scherrer, et al. (1988) J Bacteriol 170:3321-3326) and were more resistant to quinolone antibiotics that target DNA gyrase and topoisomerase (Falla, et al. (1998) Antimicrob Agents Chemother 42:3282-3284). Even more strikingly, the hip mutation protected htpR cells deficient in induction of heat shock proteins from killing by increased temperature (Scherrer, et

al. (1988) J Bacteriol 170:3321-3326). In this case, hip mutation conferred the highest degree of protection - htpR cells decreased by 2 log after a short incubation at 42□C, while virtually no decrease was observed in htpR,hipA cells. Mutants in hipAB did not protect cells from kanamycin, another cidal factor that was tested. Two explanations were offered to account for high survival of persistors. Koch suggested that survivors of lethal treatments are "dormant" cells present in a population (Koch, A. L. (1996) pp. 1640-1651. In F.C. Neidhardt, et al. (Eds.), Cellular and Molecular Biology. ASM Press, Washington). Alternatively, it was suggested that there is a certain stage in the cell cycle that is resistant to penicillin (Falla, et al. (1998) Antimicrob Agents Chemother 42:3282-3284; Scherrer, et al. (1988) J Bacteriol 170:3321-3326). However, it is hard to imagine a distinct developmental stage that occupies a 10-6 part of the cell cycle (cf. the rate of persistors to penicillin), which would mean that it has a duration of =30(min)/106 = 1.8(msec). The fact that a mutation can increase the rate of persistors to 100% (as in the case of temperature resistance) suggests that persistors are not dormant and do not represent a special stage of the cell cycle. Rather, the study of hip genes shows that E. coli has a way to dramatically increase survival in the presence of a variety of unrelated lethal factors, but the majority of cells "choose" to die. hip mutants have not been found among natural E. coli isolates, suggesting, paradoxically, that improved survival to lethal factors is a deleterious trait. This paradox suggests that the ability to eliminate defective cells (through programmed death) provides a clonal population with a significant competitive advantage.

Hip genes apparently have the potential to strongly influence the "setting" of the cell death pathway, but unfortunately their physiological role (if any) remains unknown. We shall next consider E. coli genes that are good candidates for participation in programmed death, and whose function is well understood.

DNA damage by mutagens (which include quinolone antibiotics) is sensed by the RecA protein, which becomes activated and induces hydrolysis and inactivation of the LexA repressor (Walker, G. C. (1996) pp. 1400-1416. In Neidhardt, et al. (Eds.) Cellular and Molecular Biology. ASM Press, Washington). This releases LexA from the promoter regions of a number of lex-box genes, and allows for the expression of components of the SOS DNA repair response. LexA repressor inactivation also leads to the synthesis of a rapidly hydrolyzed SulA protein that inhibits cell division by binding to FtsZ, the protein that forms the division ring. SulA therefore acts as a checkpoint - it accumulates after exposure to DNA damaging agents and inhibits cell division. Subsequently, upon DNA repair SulA is degraded by the Lon protease, and cell division proceeds. This scenario suggests that without SulA, DNA will not be properly repaired prior to replication, leading to production of non-viable cells. Interestingly, this is not the case - a

sulA mutant of an otherwise normal strain (a lon+ background) had a 1,000 fold (!) higher survival to killing by mutagenic quinolones (Piddock, et al. (1992) Antimicrob Agents Chemother 36:819-825). This experiment strongly suggests that the main role of SulA is not to aid repair, but to trigger elimination of cells with serious defects in DNA. sulA mutants have an enormous survival advantage over the wild type, yet the immediate benefit of greater survival of sulA cells is apparently outweighed by the longer-term disadvantage due to the loss of the ability to eliminate defective cells. This analysis indicates just how important programmed death might be in the life of a bacterial population.

The SulA-dependent death is strikingly similar to the way animal cells respond to mutagens - DNA damage induces repair enzymes, and also triggers accumulation of the p53 protein, which may initiate an apoptotic program. Similarly to sulA mutants, cells deficient in p53 survive DNA damage much better than the wild type (Sionov, et al. (1999) Oncogene 18:6145-6157).

While SulA appears to activate death in response to DNA damage, expression of mar genes was found to suppress killing of E. coli by mutagenic quinolones (Goldman, et al. (1996) Antimicrob Agents Chemother 40:1266-1269). Originally, the global regulator MarA was found to control expression of genes responsible for multiple resistance to antimicrobials. MarA inhibits the synthesis of the large OmpF porin and simultaneously activates expression of the AcrAB-TolC MDR pump, which results in increased efflux of toxins across the outer membrane (Okusu, et al. (1996) J. Bacteriol. 178:306-308). In order to compare killing by quinolones of uninduced cells and cells expressing mar genes, each sample was tested with a corresponding minimal concentration of antibiotic completely inhibiting growth (MIC; note that MIC was considerably higher in the case of mar expressing cells). The probability of survival of cells expressing Mar proteins was increased 100 fold (Goldman, et al. (1996) Antimicrob Agents Chemother 40:1266-1269). Killing by the other type of factors tested in this study - ß-lactam antibiotics - was unaffected by the mar genes. It appears that mar genes have a dual function of inducing antibiotic resistance mechanisms, and suppressing a programmed death response triggered by damaged DNA. The fate of cells damaged by mutagens could depend on the balance of SulA and Mar activities.

Another important locus affecting cell survival is relA. It is well established that tolerance to killing by a wide variety of factors (virtually all cidal antibiotics, for example) correlates inversely with growth rate. Slow growth activates the RelA-dependent synthesis of ppGpp which inhibits anabolic processes in bacterial cells (Cashel, et al. (1996) pp. 1400-1416. In Neidhardt, et al. (Eds.) Cellular and Molecular Biology. ASM Press, Washington).

Interestingly, ppGpp suppressed the activity of a major E. coli autolysin, SLT (Betzner, et al. (1990) FEMS Microbiol Lett 55:161-164) which would make the cells more resistant to autolysis and could explain the mechanism of tolerance to antibiotics in slow growing cells (note that an increase in ppGpp was also implicated in cell death controlled by the maz locus in an experimental model (Aizenman, et al. (1996) [published erratum appears in Proc Natl Acad Sci U S A 1996 Sep 3;93(18):9991]. Proc Natl Acad Sci U S A 93:6059-6063), as discussed in the Specialized Killer Genes section). A mutation in relA, the gene coding for ppGpp synthase, while not affecting growth rate, made non-growing cells sensitive to killing by antibiotics that inhibit cell wall synthesis (Rodionov, et al. (1995) J Bacteriol 177:4224-422980). ppGpp inhibits peptidoglycan synthesis, which complicates interpretation of this finding. It would be interesting to learn whether relA mutants also become sensitive to killing by other types of lethal factors that do not target the cell wall. RelA is a potentially very interesting cell death regulator, since homologs of RelA have been found in all bacteria so far, and all species studied become tolerant to killing when growth rate decreases. Suppression of cell death by ppGpp probably allows the cell to avoid mistaking a starvation state for an unrepairable defect.

Cell survival is also modulated by heat shock proteins that function as molecular chaperones in refolding and degradation of damaged polypeptides. Induction of heat shock proteins suppressed autolysis of E. coli by a number of ß-lactams that inhibit the synthesis of peptidoglycan (Powell, et al. (1991) J Bacteriol 173:4021-4026). This inhibition was also observed in a relA background, indicating that the effect was independent of a possible activation of a stringent response. Interestingly, induction of heat shock specifically inhibits apoptosis of animal cells by Hsp72 preventing activation of c-Jun kinase, JNK (Yaglom, et al. (1999) J Biol Chem 274:20223-20228). This regulatory activity of Hsp72 is unrelated to its function as a molecular chaperone. It seems that in bacteria, heat shock proteins can also block the programmed death of damaged cells.

The immediate cause of death in bacteria is often autolysis, and autolytic enzymes are the likely ultimate targets for possible programmed death pathways. In S. pneumoniae, mutation of the LytA autolysin prevents autolysis and causes tolerance to killing by antibiotics inhibiting cell wall synthesis (Tomasz, et al. (1970) Nature 227:138-140). The recent finding of the VncS kinase regulating autolysis in response to antibiotics in S. pneumoniae will undoubtedly facilitate the identification of a pathway linking damage to autolysin. A particular autolysin tied to killing factors has not yet been identified in gram negative species. Note that unrelated cidal antibiotics (ß-lactams, aminoglycosides, fluoroquinolones) may trigger autolysis in gram negative bacteria (Crosby, et al. (1994) J Med Microbiol 40:23-3021; Gilleland, et al. (1989) J Med Microbiol

29:41-50; Kadurugamuwa, et al. (1997) J Antimicrob Chemother 40:615-621; Vincent, et al. (1991) Antimicrob Agents Chemother 35:1381-1385), although inhibitors of peptidoglycan synthesis produce the most dramatic and complete hydrolysis of the cell wall. It is important to note that even cell wall inhibitors do not always produce a clear-cut picture of lysis when they kill bacteria (Fujimoto, et al. (1998) J Bacteriol 180:3724-3726), including cases when killing does depend on the presence of a functional autolysin (Tomasz, et al. (1970) Nature 227:138-140). It is possible that in some instances lysis is limited and localized; it is also possible that cells might die independently of lysis or autolysins, although this is not known. Several autolysins have been identified in E. coli, and two of them seem to be good candidates for a role in programmed cell death. SLT autolysin controlled by ppGpp has been mentioned above. AmiB is a particularly interesting E. coli autolysin (Tsui, et al. (1996) J Bacteriol 178:5719-5731) whose overexpression sensitizes cells to autolysis by cell-wall inhibitors. An amiB mutant had no discernible phenotype (tolerance to antibiotics has not been tested). amiB forms a "super-operon" with several genes, including mutL, a mismatch repair protein that protects from mutagens, and hfq, a global regulator that protects cells from stresses including high temperature and oxidants (apparently through activating expression of the stationary state sigma factor). It thus appears that this super-operon harbors elements that can either protect the cell from lethal factors (heat, oxidants and mutagens), or cause cell death if repair is insufficient to control the damage. The linking of repair and death elements in one operon is suggestive of a program that determines cell fate.

Altruism, cheaters and mutation rate

Altruistic suicide such as PCD, and any form of altruism for that matter, is open to subversion by egoists (cheaters) arising within the population of altruists (see (Lewis, K. (1998) J Theor Biol 193:359-363) for a discussion of altruism and its origins). Recently, mutants of M. xanthus were described that are partially defective in sporulation, but produce more spores than the wild type when added at a low level to a wild type culture (Velicer, et al. (2000) Nature 404:598-60199). Conversion to this asocial phenotype occurred spontaneously by culturing cells for 1,000 generations in the absence of conditions for fruiting body formation. Some defined single-locus mutants of M. xanthus deficient in production of A- and C-signals (asgE and asgB) also showed this "cheater" phenotype - decreased spore formation in pure culture, and superior spore production when mixed with the wild type. The ability of these mutants to lyse was not examined, but the simplest possibility seems to be lack of lysis - this would account both for superior spore production in a mixed culture, and for decreased sporulation in pure culture.

In S. pneumoniae, rare natural isolates of mutants in vncS that are resistant to autolysis have been described (Novak, et al. (1999) Nature 399:590-593). Similarly, one may expect egoistic mutants such as sulA or hip to arise and provide immediate benefit to the cell. The question then arises as to how an altruistic community is maintained. It is clear that colonies of egoists will loose out in competition with altruists who practice mutually-beneficial cooperative behavior. The overall outcome of this selection will depend on the relative rate of mutation versus the rate of elimination of egoist colonies through group selection. Thus a low mutation rate might become the principal factor in defending a population of altruists against a takeover by egoists.

We must note that a low mutation rate alone may not be sufficient to keep the egoists at bay. Let us consider a more complex scenario - two bacterial clones meet and merge. If this mixing occured frequently, the advantage of benefiting from living among altruists could outweigh the disadvantage of being outcompeted in group selection when the egoists formed their own clones. This parasitism would then severely undermine the advantage of altruistic suicide. A solution to this problem is emergence of incompatibility strategies. In bacteria, adjacent clones do not usually merge, creating a distinct empty zone between them which is especially apparent on semi-solid media (Budrene, E. O. (1985) Dokl Akad Nauk SSSR 283:470-473). Colonies also cover themselves with a layer of polysaccharide and form biofilms that are hard to penetrate. The next step of this segregation would be the formation of a more permanent clone - a multicellular organism. Permanent isolation effectively prevents a clone from invasion by egoists.

Hostile takeover at stationary state and preventive suicide

What do bacterial cells die of if they are not damaged by a harsh environment and arrive unscathed at stationary state? When cells reach stationary state, growth practically ceases. In model experiments with E. coli cultured in broth medium, the population declined to about 1% of its original size after several days in stationary state. Thereafter, the size of the population stabilized (Tormo, et al. (1990) J Bacteriol 172:4339-4347; Zambrano, et al. (1993). Science 259:1757-1760). However, detailed analysis has shown that these populations undergo a fairly complete transformation. Mutant cells that proliferated faster under these conditions, increased in numbers and took over the population by day 4-5 (Zambrano, et al. (1993) Science 259:1757-1760). Continued incubation led to accumulation of multiple mutations in these cells, further increasing their "competitive edge" at stationary state. The first GASP (growth advantage in stationary phase) mutation occurring in these cells usually creates an allele of the stationary state sigma factor rpoS (rpoS knockout does not produce a survivor phenotype). This mutation

among other things leads to a decreased production of catalase, an enzyme specifically induced under stationary state conditions. (Note that reduction in catalase will act as a mutator, further increasing the probability of additional mutations arising in this variant). The GASP mutants were found to grow faster both in stationary state and in log phase in an amino acid medium (Zinser, et al. (1999) J Bacteriol 181:5800-5807).

It appears that proliferating mutants arising at stationary state are dead-end variants that lead to a hostile takeover, resulting in the death of the population. Indeed, a GASP mutant is likely to lose out to a wild type once conditions change to low amino acid/high carbohydrate, for example. Similarly, an rpoS mutant unable to upregulate catalase at the onset of stationary state will be at a disadvantage in comparison to the wild type. Abrupt anaerobic-aerobic shift is clearly part of a regular sequence of events experienced by the intestinal E. coli. The GASP mutants are thus analogous to cancer cells - they outcompete the wild type, but are likely to ultimately die out without leaving progeny. It is quite possible that the main danger unicellular organisms face are not competitors, pathogens or lack of nutrients, but their own kin turning into "unhopeful monsters" causing death of the population. Any population surviving to stationary state faces the danger, and it is reasonable to expect that bacteria evolved counter-measures to limit its impact. A low rate of mutations would obviously help to decrease the probability of takeover mutants appearing in a stationary state population.

As in animal cells, low mutation rate alone can only postpone, but not prevent the ultimate takeover of a stationary population by deleterious mutants. Apoptosis of rapidly proliferating mutants is an additional mechanism preventing cancer in animal cells, and it is possible that programmed death also helps eliminate bacterial mutants that rapidly divide at stationary state. Cells in stationary state find themselves under unfavorable conditions, where the presence of toxic compounds produced by self and other species is increased. Virtually non-growing wild type cells will become tolerant to killing by lethal factors such as antibiotics produced by neighboring species (recall that tolerance correlates inversely with growth rate), while faster growing takeover mutants will have an active death program and will be susceptible to killing.

It is possible that unlike multicellular organisms, bacteria came up with an additional unique strategy to prevent takeover of a population by opportunistic mutants. We have discussed the case of S. pneumoniae that undergo lysis which enables genetic exchange. This is an extravagantly wasteful approach to sex - conjugation might have produced the same result without sacrificing cells. It is especially puzzling that competence for DNA transformation is a transient property of late-log cells that precedes massive autolysis occurring at stationary state

(Alloing, et al. (1998) Mol Microbiol 29:75-83). It seems that this massive suicide per se could actually be the main function of spontaneous autolysis. Once the population density becomes dangerously high and likely to produce takeover mutants, a quorum-sensing mechanism triggers lysis. This strategy is essentially preventive suicide. Paradoxically, a smaller, mutant-free population entering stationary state might have a far better chance of ultimately surviving (= producing viable progeny) than a much larger population burdened by mutant cells. For example, a typical clone of 108 cells could be reduced by autolysis to about 103 cells, and given a mutation rate of 10-7, this small population entering stationary state is likely to be free of takeover mutants, and is unlikely to create them. DNA exchange probably evolved as a secondary function of lysis, building upon a preexisting process of preventive suicide. Indeed, decrease in viability upon entering stationary state is common among bacteria, but spontaneous DNA transformation is not. As noted above, an E. coli population rapidly diminishes to 1% in size shortly after entering stationary state (it is not clear whether this death is due to autolysis). E. coli do not have spontaneous DNA transformation. This 100 fold decrease in numbers might represent preventive suicide as well.

Mutation rates in asocial populations

Is deterrence of opportunistic mutants a driving force for a low mutation rate that evolved in microorganisms? Such a causative link clearly exists in a very different system - a low mutation rate allows animals to avoid cancer for years and even decades. We must note that a role for the very low mutation rate observed in bacteria has not been determined (common explanations like "a low mutation rate is important to maintain the integrity of the genome" are not particularly illuminating). The mutation rate in E. coli is around 10-7 per gene per cell per generation. Systems like proofreading, DNA repair, as well as programmed death of cells with DNA defects, as discussed above ensure that mutation rate is kept at this extremely low level. As a result, a population of 107 cells of a species with 5, 000 genes will only produce about 5,000 mutants, each having approximately only one mutation per genome. A low mutation rate produces the immediate benefit of preventing formation of defective cells, thus a population with a lower mutation rate might grow faster. However, given the very low probability of mutations, it does not seem like a population would gain a useful selective advantage from keeping the death rate from mutations at =0.05%. Predation, host attack, disease (phage) and lethal environmental factors are likely to claim significantly more than 0.05% of a bacterial population in vivo. A replication and DNA maintenance system that worked faster, cheaper and produced more mutants would seem like a better option. It appears therefore that there must be an important reason for a low mutation rate, and decreasing the probability at which egotistic cells arise would be a strong contender.

If low mutation rates evolved to check the appearance of asocial mutants, be it cheaters that do not exhibit PCD, or takeover mutants that lead to the death of a stationary state population, one would expect that growth under asocial conditions should lead to an increased rate of mutations. This is indeed the case - among 12 clones of E. coli reinoculated daily for 10, 000 generations, three populations evolved into mutator strains with defects in mismatch repair (Sniegowski, et al. (1997) Nature 387:703-705). The mutation rate of the mutator strains increased by 2 to 3 orders of magnitude. Note that a very simple minimal glucose medium was used in the experiment, and it is possible that a more complex, or variable environment would result in mutators evolving in all populations. When the environment gave mutants a distinct advantage, reversion to a Lac+ phenotype on a lactose medium, the frequency of mutator generation by an E. coli lac population was increased 500 fold (Mao, et al. (1997) J Bacteriol 179:417-422). This experiment was also carried out in the absence of a prolonged stationary state, or conditions where PCD would be advantageous to the population. It appears that without the need for selection against asocial mutants, a higher mutation rate gives cells a distinct advantage.

Death and Survival in Biofilms

One of the most dramatic documented cases of bacterial survival is observed in biofilms. It is important to stress that biofilm cells are not necessarily more resistant to antimicrobial agents, if resistance is defined in a generally accepted way as the ability to grow in the presence of a test substance. What biofilms are good at is survival in the presence of extremely high concentrations of bactericidal antimicrobials. Genetics of biofilm development is an area of intense research (Davies, et al. (1998). Science 280:295-298; O'Toole, et al. (1999) Methods Enzymol 310:91-109; Pratt, et al. (1999) Curr Opin Microbiol 2:598-603), but little is known about the mechanism of biofilm survival.

A number of factors have been considered for the resilience of biofilm cells - the presence of a diffusion barrier to antimicrobials formed by the biofilm glycocalix; slow rate of cell growth; perhaps expression of certain resistance genes (Costerton, et al. (1999) Science 284:1318-1322). Some antibiotics like vancomycin and fluoroquinolones were shown to freely diffuse into biofilms (Darouiche, et al. (1994) J. Infect. Dis 170:720-723; Nichols, et al. (1989) J. Gen. Microbiol. 135:1291-1303; Stewart, P. S. (1996) Agents Chemother 40:2517-2522). Slower growth will result in "tolerance", a decreased susceptibility to killing by antibiotics, possibly through a sarA-dependent suppression of PCD, as described in previous sections. This will definitely contribute to survival of biofilm cells, but the observed resistance of biofilms to killing can be higher than in non-growing planktonic cell cultures (Brooun, et al. (2000) Antimicrob

Agents Chemother 44:640-646). The expression of resistance genes in a biofilm is an open question.

We recently examined dose-response killing of P. aeruginosa biofilms and found that fluoroquinolones produce a distinctly biphasic killing - at low concentrations, most of the population dies, and the remaining fraction (a decrease of 3-5 log) is completely resistant to further increase of antibiotic (Brooun, et al. (2000) Antimicrob Agents Chemother 44:640-646). The planktonic population did not produce noticeable numbers of persistors under these conditions. The surviving biofilm cells are not mutants, but rather persistor variants of the wild type. Surprisingly, the role of persistors in biofilm survival has not been considered. Yet several papers describe experiments that show killing patterns very similar to our findings of persistor cells in P. aeruginosa biofilms. In E. coli, increasing concentrations of ciprofloxacin or imipenem caused an initial decrease in live cells of a biofilm by 2-3 orders of magnitude, while the remaining small population was essentially insensitive to further increase in drug concentration (Ashby, et al. (1994) J Antimicrob Chemother 33:443-452). This pattern was also observed with amoxicillin and clindamycin in L. acidophilus, and with erythromycin and metronidazole in the case of G. vaginalis biofilms, where initial rapid killing was followed by a plateau of resistant cells (Muli, et al. (1998) J Med Microbiol 47:401-405).

It appears that biofilm survival can be largely explained by the increased production of persistor cells. In planktonic cells treated with an antibiotic, a small residual population of persistors will be eliminated by the immune system and does not present a clinical problem (it can be a problem in immunocompromised individuals). Elimination of remaining persistors by the immune system has not been specifically studied, but we know very well that the immune system is capable of eliminating an entire population of pathogens that would otherwise persist in the presence of a bacteriostatic antibiotic. The capability of the immune system is the reason for the recommended MBC (minimal bactericidal concentration) to be defined as a concentration of antibiotic that causes a drop of =99.9% of cells. Unlike planktonic cells, biofilm cells are protected from the immune system by a polysaccharide matrix (Hoyle, et al. (1990) J Antimicrob Chemother 26:1-5), and remaining persistors will be responsible for biofilm regrowth when antibiotic concentration drops, or when the treatment is discontinued based on the apparent absence of infection. This explains why it is so hard to eradicate a biofilm. At the same time, it is clear that the ability of biofilms to produce increased numbers of persistors predates our use of antibiotics (it is also possible that in some cases, biofilms do not produce more persistors than a planktonic population, and will still be more resistant to antibiotic treatment in vivo because of the protection from the immune system). Perhaps bacteria have two main life-styles - planktonic

cells for rapid proliferation and spread into new territories, and biofilms, slower growing populations focused on perseverance that use increased production of persistors as the main mechanism of survival.

The role of persistors in both planktonic and biofilm populations might be very significant. According to the currently accepted view, survival of (non-sporulating) bacterial populations is explained by two types of mechanisms - (1) induction of stress-responses (SOS, heat shock, oxidation stress, etc.), or (2) genetic mechanisms, such as appearance of resistant mutants, or acquisition of foreign genes carrying resistance elements. However, a sudden challenge by a lethal factor will not allow for the expression of a stress response. For example, a sudden rise in temperature will not allow for expression of heat shock proteins, and the majority of cells will die. Persistors do not need time for inducing resistance, and can survive a sudden challenge by a killing factor. Persistors arise at a considerably higher rate (by 1-4 orders of magnitude) than mutants. It seems likely that the process of non-heritable variation that produces persistors plays a significant role in survival of bacterial populations encountering damaging factors. Antibiotic-resistant strains have become a major clinical problem (Levy, S. B. (1998) Sci. Amer. 278:46-53), and persistors, especially those found in biofilm cells, may be important intermediates in the development of resistance.

Specialized Killer Genes

Suicide of a cell can also be directed by parasitic DNA. The demarcation between the two types of suicide may be blurred, and we will briefly consider the case of specialized killer genes (See (Engelberg-Kulka, et al. (1999) Annual Review of Microbiology 53:43-70; Gerdes, K. (2000) J Bacteriol 182:561-572) for detailed reviews).

A number of bacterial plasmids harbor toxin-antitoxin coding genes that are part of the plasmid maintenance mechanism. These genes/proteins belong to several classes, but the overall design of the suicide strategy is similar (Gerdes, K. (2000) J Bacteriol 182:561-572). A well-studied example is maintenance of the E. coli F plasmid (Hiraga, et al. (1986) J Bacteriol 166:100-104). The ccdB gene codes for a cidal toxin that inhibits DNA gyrase, while ccdA located in the same operon codes for an antitoxin. CcdA is rapidly degraded by the Lon protease, and if a daughter cell does not receive a copy of the F plasmid, it will be killed by the stable CcdB toxin.

Two toxin-antitoxin loci, chpA (mazEF) and chpB homologous to parD/pem of plasmids R1/R100 were identified in the E. coli chromosome (Masuda, et al. (1993) J Bacteriol 175:6850-6856). The mazEF genes were studied in detail, and when expressed, recombinant

MazF appeared to be toxic in the absence of MazE (Aizenman, et al. (1996) [published erratum appears in Proc Natl Acad Sci U S A 1996 Sep 3;93(18):9991]. Proc Natl Acad Sci U S A 93:6059-6063, Engelberg-Kulka, (1998) Proc Natl Acad Sci U S A 95:15481-15486). The mazEF genes are located immediately downstream of relA. It was shown that a sudden increase in ppGpp triggered by overexpression of recombinant RelA from a controllable promoter results in cell death that is partially dependent upon the presence of a mazEF locus (Aizenman, et al. (1996) [published erratum appears in Proc Natl Acad Sci U S A 1996 Sep 3;93(18):9991]. Proc Natl Acad Sci U S A 93:6059-6063). The physiological role of this locus remains unclear. Another E. coli locus, relB, originally described as a possible stringent response control element (mutations in relB relax stringent control (Cashel, et al. (1996) The Stringent Response, pp. 1400-1416. In Neidhardt, et al. (Eds.) Cellular and Molecular Biology. ASM Press, Washington), was found to code for relBE genes that are homologous to a ccd-type toxin-antitoxin system. RelE acts as a toxin which is neutralized by RelB (Gotfredsen, et al. (1998) Mol Microbiol 29:1065-1076). It is not known if RelB has a physiological function. It would be interesting to learn, for example, whether a strain with deletion in both relB and relE were affected in stringent response or had any apparent phenotype. Close homologs of RelE and RelB were found in an E. coli plasmid P307, where they are responsible for plasmid maintenance. Homologs of RelB and RelE were also found to be widely spread among bacteria, including gram positive, gram negative species, and Archaea. The role of chromosomal RelBE elements remains unclear. Recently, it was reported that E. coli K12 carries 5 distinct loci belonging to the hok class of toxin-antitoxins. All 5 are inactivated by insertional elements or by mutations.

The E. coli hipAB locus reviewed in this paper resembles a toxin-antitoxin system, although it does not have apparent homologs in GenBank. Unlike typical toxin-antitoxin genes, the hipAB locus has the potential of both killing the cell and improving survival from lethal factors. It seems that the cell could make use of its arsenal of toxin-antitoxin proteins, but the example of non-functional hok genes makes this possibility uncertain.

While suicide directed by plasmids is clearly detrimental to the host and beneficial to the parasite, suicide directed by some prophages does appear to benefit the host as well (Review, (Snyder, L. (1995) Mol Microbiol 15:415-420.)). Prophages present in the chromosome of some E. coli strains activate expression of a toxin if the host is infected by another phage. For example, a Lit protein of an E. coli prophage e14 interacts with the product of gol gene of invading phage T4, resulting in a toxin that cleaves elongation factor Tu (EF-Tu), leading to cell death and exclusion of the phage (Bergsland, et al. (1990) J Mol Biol 213:477-494; Yu, et al. (1994) Proc

Natl Acad Sci U S A 91:802-806). Programmed suicide in this case seems to have originated in the e14 prophage and represents a result of a competition between two types of parasitic DNA, with the host as a battlefield. The prophage-coded toxin limits the spread of the phage infection that will benefit both the parasitic prophage DNA and the host it permanently inhabits.

Bacteria, mitochondria, and eukaryotic PCD - in search of homologies

Bacteria are ancestral to mitochondria which play an important role in the apoptotic pathway of animal cells (Reviewed in (Budihardjo, et al. (1999) Annu Rev Cell Dev Biol 15:269-290; Crompton, M. (1999) Biochem J 341:233-249; Jacotot, et al. (1999) Ann N Y Acad Sci 887:18-30; Thress, et al. (1999) J Bioenerg Biomembr 31:321-326)). It is not known whether mitochondria have a role in apoptosis of unicellular eukaryotes. But is there a similarity between apoptotic processes observed in mitochondria and death of their bacterial ancestors? It is known that ROS activate the opening of the large permeability transition pore (PTP) in the inner mitochondrial membrane, which causes swelling, rupture of the outer membrane and the release into the cytoplasm of several intermembrane proteins - cytochrome C, procaspase 9 and Aif-1. Cytochrome C forms a complex with cytosolic Apaf-1 and procaspase 9, producing active caspase 9. This in turn activates procaspase 3 which is one of the terminal caspases of apoptosis. Aif-1 goes directly to the nucleus and activates DNA degradation. The pro- and antiapoptotic Bax and Bcl-2 mentioned above are mitochondrial proteins that apparently modify the activity of PTP. In the process of releasing apoptotic proteins, mitochondria are destroyed. This destruction does not appear to be related to autolysis of bacterial cells. At the same time, bacteria harbor large stretch-activated channels in the cytoplasmic membrane that open in response to a sudden decrease in osmolarity of the environment (Levina, et al. (1999) Embo J 18:1730-1737; Sukharev, et al. (1994) Nature 368:265-268). The opened channels allow solutes to rapidly escape from the cell, relieving osmotic pressure. Known bacterial channels are not homologous to the mitochondrial PTP. It is conceivable that the channels might also play a role in programmed death of bacterial cells, but there are currently no data to support this.

Several eukaryotic apoptotic proteins have homologs in bacteria (Aravind, et al. (1999) Trends in Biochemical Sciences 24:47-53). Whole genome searching shows that an "apoptotic" Ap-ATP domain found in several apoptotic proteins including Apaf-1 is present in B. subtilis and actinomycetes. A TIR domain responsible for interaction of some apoptotic proteins shows up in Streptomyces, B. subtilis, Synechocystis sp., and Rhizobium, and caspase homologs are found in Streptomyces. In the absence of functional data it is not possible to decide whether these proteins are involved in PCD in bacteria, or do other useful things. However, the presence

of apoptotic protein homologs in only several bacteria would argue against the conservation of a homologous PCD among pro- and eukaryotes.

Little is known about death in Archaea whose physiology and life-style closely resemble those of eubacteria. As we have already mentioned, RelBE-type toxin-antitoxin specialized suicide genes are found in the genomes of Archaea (Pedersen, et al. (1999). Mol Microbiol 32:1090-1102), but their role is unknown. Whether organisms from all domains of life have programmed elimination of defective cells remains an intriguing open question.

4.5. Tolerance Gene Antagonists and Intolerance Gene Agonists

In preferred embodiments, the invention provides tolerance gene antagonists which may be a nucleic acid antagonist of the tolerance gene or a small molecule, and preferably a peptide antagonist, of the tolerance gene protein or polypeptide product as outlined below. The below-described compositions and methods are offered as examples of what is available to the skilled artisan and are not exclusive of other widely known methods, such as widely used methods of combinatorial chemistry available using small molecule libraries and microarrays to identify and isolate small molecule antagonists as well as agonists (e.g. for use in inhibiting a microbial intolerance gene target).

4.5.1. Antisense, Ribozyme and Triplex Techniques

One aspect of the invention relates to the use of the isolated nucleic acid in "antisense" therapy. As used herein, "antisense" therapy refers to administration or in situ generation of oligonucleotide molecules or their derivatives which specifically hybridize (e.g. bind) under cellular conditions, with the cellular mRNA and/or genomic DNA encoding one or more of the subject tolerance gene proteins so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a tolerance gene protein. Alternatively, the antisense construct is an oligonucleotide probe which is generated ex vivo and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a tolerance gene. Such oligonucleotide probes are

preferably modified oligonucleotides which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) Biotechniques 6:958-976; and Stein et al. (1988) Cancer Res 48:2659-2668. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between the -10 and +10 regions of the tolerance gene nucleotide sequence of interest, are preferred.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to tolerance gene mRNA. The antisense oligonucleotides will bind to the tolerance gene mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. a sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently been shown to be effective at inhibiting translation of mRNAs as well. (Wagner, R. 1994. Nature 372:333). Therefore, oligonucleotides complementary to either the 5' or 3' untranslated, non-coding regions of a tolerance gene could be used in an antisense approach to inhibit translation of endogenous tolerance gene mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region oftolerance genemRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In certain embodiments, the

oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides, or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that in vitro studies are first performed to quantitate the ability of the antisense oligonucleotide to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. W088/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. W089/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents. (See, e.g., Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil,

beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an *-anomeric oligonucleotide. An *-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual *-units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate olgonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

While antisense nucleotides complementary to the tolerance gene coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred.

The antisense molecules should be delivered to cells which express the tolerance gene in vivo. A number of methods have been developed for delivering antisense DNA or RNA to cells; e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired cells (e.g., antisense linked to peptides or antibodies

that specifically bind receptors or antigens expressed on the target cell surface) can be administered systematically.

However, it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs. Therefore a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous tolerance gene transcripts and thereby prevent translation of the tolerance gene mRNA. For example, a vector can be introduced in vivo such that it is taken up by a cell and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al, 1982, Nature 296:39-42), etc. Any type of plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site; e.g., the choroid plexus or hypothalamus. Alternatively, viral vectors can be used which selectively infect the desired tissue; (e.g., for brain, herpesvirus vectors may be used), in which case administration may be accomplished by another route (e.g., systematically).

Ribozyme molecules designed to catalytically cleave tolerance gene mRNA transcripts can also be used to prevent translation of tolerance gene mRNA and expression of tolerance gene. (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy tolerance gene mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and

production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591. There are hundreds of potential hammerhead ribozyme cleavage sites within the nucleotide sequence of human tolerance gene cDNA. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of thetolerance genemRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in Tetrahymena Thermophila (known as the IVS, or L-19 IVS RNA) and which has been extensively described by Thomas Cech and collaborators (Zaug, et al., 1984, Science, 224:574-578; Zaug and Cech, 1986, Science, 231:470-475; Zaug, et al., 1986, Nature, 324:429-433; published International patent application No. WO88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in tolerance gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express the tolerance gene in vivo e.g., hypothalamus and/or the choroid plexus. A preferred method of delivery involves using a DNA construct "encoding" the robozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous tolerance gene messages and inhibit translation. Because ribozymes unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous tolerance gene expression can also be reduced by inactivating or "knocking out" the tolerance gene or its promoter using targeted homologous recombination. (E.g., see Smithies et al., 1985, Nature 317:230-234; Thomas & Capecchi, 1987, Cell 51:503-512; Thompson et al., 1989 Cell 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional tolerance gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous tolerance gene (either the coding regions or regulatory regions of the tolerance gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express tolerance gene in vivo. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the tolerance gene. Such approaches are particularly suited in the agricultural

field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive tolerance gene (e.g., see Thomas & Capecchi 1987 and Thompson 1989, supra). However this approach can be adapted for use in humans provided the recominant DNA constructs are directly administered or targeted to the required site in vivo using appropriate viral vectors, e.g., herpes virus vectors for delivery to brain tissue; e.g., the hypothalamus and/or choroid plexus.

Alternatively, endogenous tolerance gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the tolerance gene (i.e., the tolerance genepromoter and/or enhancers) to form triple helical structures that prevent transcription of the tolerance gene in target cells in the body. (See generally, Helene, C. 1991, Anticancer Drug Des., 6(6):569-84; Helene, C., et al., 1992, Ann, N.Y. Accad. Sci., 660:27-36; and Maher, L.J., 1992, Bioassays 14(12):807-15).

Likewise, the antisense constructs of the present invention, by antagonizing the normal biological activity of one of the tolerance geneproteins, can be used in the manipulation of tissue, e.g. tissue differentiation, both in vivo and for ex vivo tissue cultures.

Furthermore, the anti-sense techniques (e.g. microinjection of antisense molecules, or transfection with plasmids whose transcripts are anti-sense with regard to aGLC1AmRNA or gene sequence) can be used to investigate role of tolerance genein developmental events, as well as the normal cellular function of tolerance genein adult tissue. Such techniques can be utilized in cell culture, but can also be used in the creation of transgenic animals, as detailed below.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see U.S. Pat. No. 5,093,246, which is incorporated by reference herein in its entirety. As such within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding tolerance gene proteins.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the molecule of interest for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20

ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate sequences may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription are preferably single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides should promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

Antisense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Moreover, various well-known modifications to nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

4.5.2. Polypeptides Antagonists and Agonists of the Present Invention

The present invention also makes available isolated tolerance and intolerance gene polypeptides which are isolated from, or otherwise substantially free of other cellular proteins, especially other signal transduction factors and/or transcription factors which may normally be associated with the tolerance gene polypeptide. These tolerance and intolerance gene polypeptides may be adapted to providing the tolerance gene inhibitor and intolerance gene activatior functions of the invention using methods known in the art. In particular, these materials and methods may be adapted to isolating and identifying polypeptide antagonists of tolerance gene and peptide agonists of intolerance genes.

The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure or purified preparations" are defined as encompassing preparations of tolerance gene polypeptides having less than about 20% (by dry weight) contaminating protein, and preferably having less than about 5% contaminating protein. Functional forms of the subject polypeptides can be prepared, for the first time, as purified preparations by using a cloned gene as described herein. By "purified", it is meant, when referring to a peptide or DNA or RNA sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above. "Isolated" and "purified" do not encompass either natural materials in their native state or natural materials that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins, or chromatography reagents such as denaturing agents and polymers, e.g. acrylamide or agarose) substances or solutions. In preferred embodiments, purified tolerance gene polypeptide preparations will lack any contaminating proteins from the same animal from which tolerance gene product is normally

produced, as can be accomplished by recombinant expression of, for example, a human tolerance gene protein in a non-human cell.

Full length proteins or fragments corresponding to one or more particular motifs and/or domains or to arbitrary sizes, for example, at least 5, 10, 25, 50, 75, 100, 125, 150 amino acids in length are within the scope of the present invention.

For example, isolated tolerance gene polypeptides can include all or a portion of an amino acid sequences corresponding to a tolerance gene polypeptide. Isolated peptidyl portions of tolerance gene proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a tolerance gene polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") tolerance gene protein.

Another aspect of the present invention concerns recombinant forms of the tolerance gene proteins. Recombinant polypeptides preferred by the present invention, in addition to native tolerance gene proteins, are at least 92% homologous and more preferably 94% homologous and most preferably 95% homologous with an amino acid sequence of a tolerance gene protein. Polypeptides which are at least about 98-99% homologous with a tolerance gene sequence is also within the scope of the invention. In a preferred embodiment, a tolerance gene protein of the present invention is a tolerance gene protein. In particularly preferred embodiments, a tolerance gene protein has a tolerance gene bioactivity. In certain preferred embodiments, the invention features a purified or recombinant tolerance gene polypeptide having a molecular weight of approximately 17 kD. It will be understood that certain post-translational modifications, e.g., phosphorylation and the like, can increase the apparent molecular weight of the tolerance gene protein relative to the unmodified polypeptide chain.

The present invention further pertains to recombinant forms of one of the subject tolerance gene polypeptides which are encoded by genes derived from a mammalian organism, and which have amino acid sequences evolutionarily related to a tolerance gene protein. Such recombinant tolerance gene polypeptides preferably are capable of functioning in one of either role of an agonist or antagonist of at least one biological activity of a wild-type ("authentic")

tolerance gene protein of the appended sequence listing. The term "evolutionarily related to", with respect to amino acid sequences of tolerance gene proteins, refers to both polypeptides having amino acid sequences which have arisen naturally, and also to mutational variants of tolerance gene polypeptides which are derived, for example, by combinatorial mutagenesis. Such evolutionarily derived tolerance gene polypeptides preferred by the present invention have a tolerance gene bioactivity and are at least 92% homologous and more preferably 94% homologous and most preferably 98-99% homologous to a tolerance gene protein. In a particularly preferred embodiment, a tolerance gene protein comprises the amino acid coding sequence of sarA.

In general, polypeptides referred to herein as having an activity (e.g., are "bioactive") of a tolerance gene protein are defined as polypeptides which include an amino acid sequence corresponding (e.g., identical or homologous) to all or a portion of the amino acid sequences of a tolerance gene protein sarA and which mimic or antagonize all or a portion of the biological/biochemical activities of a naturally occurring tolerance gene protein. In preferred embodiments, the biochemical activities are related to gene expression, pituitary development, and abdominal development related to umbilical and vitelline artery expression.

Other biological activities of the subject tolerance gene proteins are described herein or will be reasonably apparent to those skilled in the art. According to the present invention, a polypeptide has biological activity if it is a specific agonist or antagonist of a naturally-occurring form of a tolerance gene protein.

The present invention further pertains to methods of producing the subject tolerance gene polypeptides. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The cells may be harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant tolerance gene polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant tolerance gene polypeptide is a fusion protein containing a domain which facilitates its purification, such as GST fusion protein or poly(His) fusion protein.

Moreover, it will be generally appreciated that, under certain circumstances, it may be advantageous to provide homologs of one of the subject tolerance gene polypeptides which function in a limited capacity as one of either a tolerance gene agonist (mimetic) or a tolerance gene antagonist, in order to promote or inhibit only a subset of the biological activities of the naturally-occurring form of the protein. Thus, specific biological effects can be elicited by treatment with a homolog of limited function, and with fewer side effects relative to treatment with agonists or antagonists which are directed to all of the biological activities of naturally occurring forms of tolerance gene proteins.

Homologs of each of the subject tolerance gene proteins can be generated by mutagenesis, such as by discrete point mutation(s), or by truncation. For instance, mutation can give rise to homologs which retain substantially the same, or merely a subset, of the biological activity of the tolerance gene polypeptide from which it was derived. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to a downstream or upstream member of the biochemical pathway, which includes the tolerance gene protein. In addition, agonistic forms of the protein may be generated which are constituatively active. Thus, the human tolerance gene protein and homologs thereof provided by the subject invention may be either positive or negative regulators of gene expression.

The recombinant tolerance gene polypeptides of the present invention also include homologs of the authentic tolerance gene proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter ubiquitination or other enzymatic targeting associated with the protein.

Tolerance gene polypeptides may also be chemically modified to create tolerance gene derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of tolerance gene proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

Modification of the structure of the subject tolerance gene polypeptides can be for such purposes as enhancing therapeutic or prophylactic efficacy, stability (e.g., ex vivo shelf life and resistance to proteolytic degradation in vivo), or post-translational modifications (e.g., to alter phosphorylation pattern of protein). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, or to produce specific antagonists thereof, are considered functional equivalents of the tolerance gene polypeptides described in more detail

herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. isosteric and/or isoelectric mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, Biochemistry, 2nd ed.; Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functionaltolerance genehomolog (e.g. functional in the sense that the resulting polypeptide mimics or antagonizes the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method for generating sets of combinatorial mutants of the subject tolerance gene proteins as well as truncation mutants, and is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in modulating gene expression. The purpose of screening such combinatorial libraries is to generate, for example, novel tolerance gene homologs which can act as either agonists or antagonist, or alternatively, possess novel activities all together.

Likewise, tolerance gene homologs can be generated by the present combinatorial approach to selectively inhibit gene expression. For instance, mutagenesis can provide tolerance gene homologs which are able to bind other signal pathway proteins (or DNA) yet prevent propagation of the signal, e.g. the homologs can be dominant negative mutants. Moreover, manipulation of certain domains of tolerance gene by the present method can provide domains more suitable for use in fusion proteins.

In one embodiment, the variegated library of tolerance gene variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential tolerance gene sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of tolerance gene sequences therein.

There are many ways by which such libraries of potential tolerance gene homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential tolerance gene sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

Likewise, a library of coding sequence fragments can be provided for a tolerance gene clone in order to generate a variegated population of tolerance gene fragments for screening and subsequent selection of bioactive fragments. A variety of techniques are known in the art for generating such libraries, including chemical synthesis. In one embodiment, a library of coding sequence fragments can be generated by (i) treating a double stranded PCR fragment of atolerance genecoding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule; (ii) denaturing the double stranded DNA; (iii) renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products; (iv) removing single stranded portions from reformed duplexes by treatment with S1 nuclease; and (v) ligating the resulting fragment library into an expression vector. By this exemplary method, an expression library can be derived which codes for N-terminal, C-terminal and internal fragments of various sizes.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for

rapid screening of the gene libraries generated by the combinatorial mutagenesis oftolerance genehomologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate tolerance gene sequences created by combinatorial mutagenesis techniques.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 1026 molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays. To overcome this problem, a new technique has been developed recently, recrusive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, PNAS USA 89:7811-7815; Yourvan et al., 1992, Parallel Problem Solving from Nature, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, Protein Engineering 6(3):327-331).

The invention also provides for reduction of the tolerance gene proteins to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a mammaliantolerance genepolypeptide of the present invention with either upstream or downstream components. Thus, such mutagenic techniques as described above are also useful to map the determinants of the tolerance gene proteins which participate in protein-protein interactions involved in, for example, binding of the subject tolerance gene polypeptide to proteins which may function upstream (including both activators and repressors of its activity) or to proteins or nucleic acids which may function downstream of the tolerance gene polypeptide, whether they are positively or negatively regulated by it. To illustrate, the critical residues of a subject tolerance gene polypeptide which are involved in molecular recognition of a component upstream or downstream of a tolerance gene can be determined and used to generate tolerance gene-derived peptidomimetics which competitively inhibit binding of the authentic tolerance gene protein with that moiety. By employing, for example, scanning mutagenesis to map the amino acid residues of each of the subject tolerance gene proteins which are involved in binding

other extracellular proteins, peptidomimetic compounds can be generated which mimic those residues of the tolerance gene protein which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of a tolerance gene protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) J Med Chem 29:295; and Ewenson et al. in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al. (1985) Tetrahedron Lett 26:647; and Sato et al. (1986) J Chem Soc Perkin Trans 1:1231), and b-aminoalcohols (Gordon et al. (1985) Biochem Biophys Res Commun126:419; and Dann et al. (1986) Biochem Biophys Res Commun 134:71).

4.6. Screening Methods for Identification of Tolerance and Intolerance Genes

The invention further provides methods for identifying additional microbial (e.g. bacterial) gene tolerance and intolerance gene targets using methods exemplified herein as well as equivalent screens, particularly those methods known for high-throughput screening, which are known in the art.

In one embodiment, the method of the invention provides a method for identifying a microbial tolerance gene target for treatment of a persistent/ tolerant microbial cell population using a library of mutated microorganisms comprising multiple independent clones. Such libaries of mutant microorganisms having tagged mutant genes, are known in the art and are typically collection derived from a single wild-type strain of the microorganism of interest. The screen may be effected by contacting a mutated clone from the library with an antibiotic agent; and comparing the survival of the mutated clone with the survival of the wild-type strain under the same conditions. If the mutated clones shows decreased survival in the presence of the antibiotic agent relative to the wild-type strain correspond to clones having a tagged mutation in a microbial tolerance gene, then the tagged gene in the mutant is a microbial tolerance gene. The corresponding tagged microbial tolerance gene may then be isolated and identified, and its encoded protein expressed, using methods known in the art (see e.g. Sambrook et al. Molecular

Cloning: A Laboratory Manual (1989) Cold Spring Harbor Press, the contents of which are incorporated by reference).

This aspect of the invention further includes methods for identifying microbial intolerance gene target for treatment of a persistent/ tolerant microbial cell population. For example, one way to identify additional microbial intolerance genes is to use a library of bacteria transformed with a bacterial gene overexpression vector library expressing a representative number of the genes from the bacterial microorganism by virtue of being functionally linked to a strong promoter. A single clone from such a library of bacteria is then contacted with an antibiotic agent and the survival of the clonal isolate is compared to an untransformed bacteria of the same strain and under the same conditions. If the clonal isolated evinces a decreased survival in the presence of the antibiotic agent relative to the untransformed bacteria of the same strain, then the corresponding overexpressed bacterial gene is a bacterial intolerance gene. The corresponding tagged microbial intolerance gene may then be isolated and identified, and its encoded protein expressed, using methods known in the art (see e.g. Sambrook et al. Molecular Cloning: A Laboratory Manual (1989) Cold Spring Harbor Press, the contents of which are incorporated by reference). Bacterial overexpression vector libraries are well known in the art.

5. Examples

5.1. Effect of the Multidrug Resistance Pumps on Biofilm Antimicrobial Resistance

We first examined the affect of the multidrug resistance gene pumps on the sensitivity to antimicrobials of bacteria growing as a biofilm. Bacterial biofilms show enormous levels of antibiotic resistance, but little is known about the underlying molecular mechanisms. Multidrug resistance pumps (MDRs) are responsible for the extrusion of chemically unrelated antimicrobials from the bacterial cell. Contribution of the MDR-mediated efflux to antibiotic resistance of *Pseudomonas aeruginosa* biofilms was examined by using strains overexpressing and lacking the MexAB-OprM pump. Resistance of *P. aeruginosa* biofilms to ofloxacin was dependent on the expression of MexAB-OprM but only in the low concentration range. Unexpectedly, biofilm resistance to ciprofloxacin, another substrate of MexAB-OprM, did not depend on the presence of this pump. Figure 1 shows the killing by ciprofloxacin of $MexAB^{++}$ and $MexAB^{-}$ cells growing as a biofilm. Dose-dependent killing indicated the presence of a small "superresistant" cell fraction. This fraction was primarily responsible for very high resistance of P. aeruginosa biofilms to quinolones. Bacterial cells recovered from a biofilm and

tested under nongrowing conditions with tobramycin exhibited higher resistance levels than planktonic cells but lower levels than cells of an intact biofilm.

5.2 Effect of sarA on Survival of *Staphylococcus aureus*

In this study, we aimed to learn whether sarA cells had a generally higher susceptibility to killing by a number of unrelated factors. We found that the sarA mutation increases sensitivity of cells to killing by penicillin, mitomycin C, and heat.

The *sarA* (Staphylococcal accessory regulator) gene of *Staphylococcus aureus* is a global regulator that controls expression of virulence factors. A mutation in *sarA* has been previously reported to increase susceptibility of cells to killing by cell-wall acting antibiotics (Fujimoto, D.F. et al. (1998), J. Bacteriol. 180:3724). We tested the possibility of SarA acting as a general regulator (suppressor) of cell death. Sensitivity of the *sarA* mutant to a variety of unrelated damaging factors was investigated. The *sarA* mutant had higher sensitivity to penicillin (a β-lactam), mitomycin C (a mutagen), and heat (60°C), with live cell numbers dropping as much as 3 orders of magnitude when compared to the wild-type strain. Persisters were completely eradicated by mitomycin in *sarA* cells, but not in the wild type. At the same time, there was no difference between the survival of *sarA* mutant and wild type strain in response to ciprofloxacin, trovafloxacin (both fluoroquinolones) and tobramycin (an aminoglycoside). These results suggest that SarA might be suppressing a branch of a cell death regulatory pathway that is activated by several unrelated damaging factors. The observed eradication of persisters by mitomycin C in a *sarA* culture is a useful model for developing drugs aimed at combating recalcitrant infections, such as biofilms, where persisters are largely responsible for population survival (see e.g., Brooun, A. et al. (2000), Antimicrob. Agents Chemother. 44:640). A combination of a substance inhibiting SarA (or a similar cell death suppressor) and a conventional cidal antibiotic would provide a fruitful approach to anti-biofilm drug development.

Materials And Methods

Bacterial strains and culture conditions *S. aureus* wild type strain RN 6390 and its isogenic ALC488 *sarA*::*ermC* mutant (Manna, A.C. et al. (1998), J. Bacteriol. 180:3828), were provided by A. L. Cheung. *S. aureus* phage 80α was provided by J. C. Lee and propagated on wild type strain RN 450 provided by R. K. Jayaswal. Tryptic soy broth (TSB) and tryptic soy agar (TSA, Difco) were used to culture the *S. aureus* strains. For killing experiments, cells were cultured in liquid TSB medium at 37°C with aeration, essentially as described in Fujimoto, D.F. et al. ((1998), J. Bacteriol. 180:3724). MIC was determined by broth microdilution according to NCCLS recommendations.

Antimicrobial agents Penicillin, mitomycin C, tobramycin and ciprofloxacin were obtained from Sigma, St. Louis, MO. Trovafloxacin was from Pfizer.

Results

We chose a set of unrelated cidal factors to investigate the role of the *sarA* mutation in cell survival. These factors included DNA damaging agents; an aminoglycoside antibiotic and high temperature. We first tested the action of penicillin, which was originally reported to produce increased killing of *sarA* cells (Fujimoto, D.F. et al. (1998) J. Bacteriol. 180:3724). An exponential phase culture of the wild type strain RN6390 and the *sarA* mutant ALC488 was challenged by penicillin at 0.4 μg/ml, ten times the minimal inhibitory concentration (MIC). Samples were withdrawn over a period of time and plated to determine the fraction of viable cells (see Fig. 2). Two hours after the treatment with penicillin, the amount of live cells dropped two fold in the wild type and decreased ten fold in the *sarA* strain. After that, the number of live cells stabilized in the wild type strain and continued to drop gradually in the *sarA* mutant. After treatment with penicillin, the *sarA* culture lysed, while little lysis was observed in the wild type. This result agrees qualitatively with previous findings (Fujimoto, D.F. et al. (1998), J. Bacteriol. 180:3724). However, in our hands the absolute level of killing by penicillin in both strains, and the extent of difference in killing between the wild type and *sarA* was lower than in the previously published study. It seemed possible that over time the *sarA* strain accumulated suppressor mutation(s), improving cell survival. We therefore transduced the *sar*::Tn551(*erm*^{r}) to the wild type RN6390 and checked the resistance of transductants to killing. The sensitivity of the transductants to killing was the same as with the original ALC488 strain. The reason for the discrepancy with published work remains unknown.

Next, survival of *sarA* cells was tested with a DNA damaging agent. Mitomycin C is a mutagen which crosslinks DNA by forming bisadducts with deoxyguanosine residues on two DNA single strand (Tomasz, M. et al. (1987), Science 235:1204). Cells were challenged with mitomycin C at 0.8 μg/ml, which is the MIC for the wild type. The MIC for the *sarA* mutant was 0.4 μg/ml. After 8 hours of incubation, there was no killing of the wild type, while the number of live *sarA* cells dropped 4 orders of magnitude (Fig. 3(a)). The *sarA* culture incubated with mitomycin exhibited strong lysis. The time-dependent killing of *sarA* cells by mitomycin is distinctly biphasic – after the majority of the population is eradicated, a subpopulation of "persisters" relatively resistant to killing survives. Persisters are responsible for the inability of antibiotics to sterilize bacterial populations, especially in biofilms (see Brooun, A. et al. (2000), Antimicrob. Agents Chemother. 44:640). It was interesting to learn whether mitomycin at higher concentrations would be able to sterilize a *S. aureus* culture carrying a *sarA* mutation. In a dose-response killing experiment (Fig. 2(b)), *sarA* cells were eliminated at 4 μg/ml, and no

persisters were seen above the detection level. At this concentration, the wild type population still had 10^5 live persister cells/ml. This experiment showed that a combination of a *sarA* mutation and a DNA-acting antibiotic can sterilize a bacterial culture. It seemed that fluoroquinolones which are also DNA-acting agents (inhibitors of DNA gyrase and topoisomerase, Hooper, D.C. (2001), Clin. Infect. Dis. 15: 59(; and are mutagenic in bacteria (Piddock, L.J. et al. (1992), Antimicrob. Agents Chemother. 36:819), could similarly be more effective in killing *sarA* cells. Unexpectedly, this was not the case. Both the wild type and the *sarA* strain showed rather similar dynamics of killing by ciprofloxacin and trovafloxacin (results not shown).

Next, we tested susceptibility of *sarA* cells to killing by an aminoglycoside antibiotic tobramycin. There was no difference between *sarA* and the wild type, and both cultures exhibited sharply biphasic killing, revealing a prominent population of persisters completely resistant to killing with the tested concentration of the antibiotic (10 times the MIC) (results not shown).

Apart from studying the action of chemical toxins, we also examined relative survival of *sarA* cells to high temperature, a physical factor used in sterilization and pasteurization. Exponential phase cells were placed in a water bath at 60°C, and samples were taken every 2 minutes for colony counting. After 2 minutes of heat treatment, there was no change in the wild type, while the number of live *sarA* cells dropped almost 100 fold (see Figure 4). The remaining persisters in *sarA* were relatively resistant to killing.

SarA is a global regulator of virulence factor expression in *S. aureus*, and a *sarA* mutation was reported to increase susceptibility of cells to killing by cell wall inhibitors (see Fujimoto, D.F. et al. (1998), J. Bacteriol. 180:3724). This observation suggested that *sarA* might be involved in the synthesis/degradation of the cell wall. Specifically, evidence points to *sarA* mutants having increased expression of a major autolysin LytM (Fujimoto, D.F. et al. (1998), J. Bacteriol. 180:3724). It appeared that a *sarA* mutation might be potentiating killing by cell wall inhibitors which requires the action of autolysins. At the same time, "autolysis-independent" killing by penicillin has also been described in gram positive species. "Cin" mutants of *S. pneumoniae,* (Moreillon, P. et al. (1990), Antimicrob. Agents Chemother. 34: 33), and similar mutants in *S. aureus* (Tomasz, A. et al. (1986), Eur. J. Clin. Microbiol. 5: 710), have been described that are killed by penicillin without lysis. It has also been noted that cell death considerably precedes lysis (Bayles, K.W. (2000), Trends Microbiol. 8: 274). An *lrgAB* locus was reported to affect killing by penicillin in *S. aureus* (Groicher, K.H. et al. (2000), J.Bacteriol. 182: 1794). A deletion in *lrgAB* caused increased killing by penicillin. It has been proposed that SarA affects penicillin killing by acting at the level of LrgAB (Bayles, K.W. (2000), Trends Microbiol. 8:274). However, the magnitude of changes in cell susceptibility to penicillin killing

observed in *lrgAB* mutants is 2-3 fold, considerably less than in *sarA* cells. This would suggest that SarA acts independently of *lrg*.

We were interested in testing the possibility of SarA acting as a general suppressor of cell death. Then, one would expect different cidal factors to have a greater effect on *sarA* cells. The damaging factors tested in this study are structurally and mechanistically distinct and act on different targets. Penicillin is a beta-lactam that inhibits bacterial cell wall synthesis, mitomycin C is a mutagen, trovafloxacin and ciprofloxacin are fluoroquinolones that target DNA gyrase/topoisomerase, tobramycin is an aminoglycoside that inhibits protein synthesis, and heat is a general damaging agent.

In these experiments, we found that *sarA* cells were more sensitive to penicillin. Additionally, *sarA* cells were considerably more susceptible to killing by high temperature and mitomycin C. The action of mitomycin C against *sarA* was especially strong – the killing was 4 orders of magnitude greater as compared to the wild type in a time-dependent experiment. Significant numbers of live wild type cells were recovered in a dose-response experiment, while the *sarA* culture was effectively sterilized. Note that the MIC of *sarA* was comparable to that of the wild type for the tested antibiotics, meaning that the mutation did not impair possible resistance mechanisms, but only affected sensitivity to killing. This conclusion is further substantiated by our observation of similar responses of *sarA* and the wild type to fluoroquinolones and tobramycin, showing that *sarA* is not merely a "weak" strain. But why does *sarA* confirm susceptibility to killing to a set of unrelated factors, rather than to all killing conditions? One possibility is that only certain factors trigger a PCD pathway that is inhibited by SarA. It is also possible that SarA controls a branch of the PCD pathway which is triggered by a number of unrelated killing factors. Of course, at this point we can not exclude other possibilities, such as SarA acting in a manner that does not involve PCD.

Irrespective of what particular model proves to be true, these results support a new avenue for antimicrobial drug development. Successful antimicrobial therapy apparently relies on the cooperation of antibiotics and the immune system. This would explain the usefulness of the empirically derived measure of killing, MBC, a concentration of an antibiotic that kills ≥99.9% of cells. Remaining persisters are probably mopped up by the immune system. However, in biofilm infections, this MBC measure becomes meaningless, since the cell population is physically protected from the immune system by the exopolysaccharide matrix (Costerton, J.W. et al. (1999), Science 284: 1318), and persister cells remaining after antibiotic treatment will resuscitate the biofilm. This would explain the relapsing nature of biofilm infections (Lewis, K. (2001), Antimicrobial Agents and Chemotherapy (in press)). One possibility is that persister cells are, at least temporarily, disabled for PCD (Lewis, K. (2000), Microbiol. Mol. Biol. Rev. 64: 503). Activating cell death would potentiate cidal antibiotics and lead to eradication of

persisters. This is what we observed in experiments with *sarA* cells treated with mitomycin C – persisters were completely eradicated. This model experiment suggests that a SarA inhibitor in combination with mitomycin should sterilize a *S. aureus* culture and points to a way of eradicating biofilm infections.

5.3. Screening Assays

We note that genes affecting tolerance will not be identified by conventional drug screening methods, such as by tests for the increased tolerance or susceptibility of growth in the presence of a particular antimicrobial agent. Such tests will only indicate that genes participating in resistance mechanisms that affect the ability of cells to grow in the presence of antimicrobials. The genes we are interested in targeting affect microbial survival, but not the ability to grow in the presence of antimicrobials. Therefore, the MIC (minimal inhibitory concentration) of such mutants is similar to MIC of the wild type (within about a 2-fold difference), while the MBC (minimal bactericidal concentration) of such mutants is significantly different from that of the corresponding wild-type strain.

Substances identified in screens for anti-tolerance compounds will have a number of useful applications. Biofilm infections are recalcitrant due to the very high tolerance of biofilm cells to killing by antimicrobials. A unique feature of the biofilm according to our data is apparently the presence of high numbers of persisting cells. The biofilm does not grow in the presence of antibiotics, and a challenge for anti-biofilm drug discovery is not to come up with new conventional antibiotics, but rather to discover those compounds which specifically target persistent/tolerant cells. Identified substances may be applied in combination with conventional bactericidal antibiotics and by preventing tolerance, should produce a fairly complete eradication of pathogens. Examples of infections that may be treated with anti-tolerance compounds include: P. aeruginosa infections in cystic fibrosis; S. aureus infections causing endocarditis; S. pneumoniae meningitidis; as well as numerous types of microbial biofilm infections of implants and indwelling devices like catheters.

The screens for identifying anti-tolerance compounds, are based upon the ability of the compound to potentiate killing by sub-inhibitory concentrations of a conventional antibiotic. This is because anti-tolerance compounds are not expected to have any activity on their own, but will only potentiate the bactericidal action of conventional antibiotics. Several formats have been examined as described below.

5.4. Lysis Potentiation

Various antibiotics cause autolysis in both gram positive and gram negative bacteria. Mutants of S. pneumoniae and S. aureus have been described (vncS, lytA, lytM) that do not lyse, and are highly resistant to killing by antibiotics. In S. aureus, a deletion of the Sar regulator that controls virulence and represses synthesis of the LytM autolysin produces cells that grow normally, have a normal MIC to antibiotics, but penicillin decreases the number of sar cells 1000 times better as compared to the wild type. We also found that the sar strain has a 50-100 fold higher sensitivity to killing by quinolones. This strain was used to validate the screen.

A fairly dense suspension of S. aureus sar in MH broth at OD of 0.3 was placed in wells of a microtiter plate. The medium contained increasing concentrations of penicillin, and strong lysis was observed at 6 ng/ml penicillin after an overnight incubation by following the OD with a microtiter plate reader. At this concentration, penicillin had no effect on lysis of the wild type. This validation experiment indicated that a putative inhibitor of Sar should cause lysis of wild type cells in the presence of 6 ng/ml penicillin.

Cells of a S. aureus wild type strain in MH broth were dispensed in microtiter plates (at OD 0.3 in the presence of 6 ng/ml penicillin) and substances from a compound library were added in the wells at 20 µg/ml. After an overnight incubation, the OD was measured, and wells with a decreased density showing lysis indicated hit compounds. The rate of primary hits was around 1%. Of these, most caused lysis or growth inhibition when tested alone. Two compounds were identified among the 15,000 tested that strongly potentiated lysis by penicillin. Specifically, compound 2 had no effect on its own at 20 µg/ml, but caused strong lysis at 2.5 µg/ml in the presence of penicillin.

Compounds identified in this screen might be inhibitors of Sar or similar components that suppress autolysis; or they may be activators of factors like VncS or LytM that activate autolysis. In either case, this screen demonstrates the feasibility of finding compounds that disable cell tolerance.

A more targeted screen using autolysin (LytM) activation

We have a LytM-LacZ fusion that allows to screen for induction of LytM in a high-throughput format using fluorescent (mum-gal) substrates. A LytM-GFP fusion could also be constructed to further simplify the screen. A mutation in sar activates LytM expression, suggesting this screen will yield inhibitors of SAR and inhibitors of any other regulator repressing LytM expression.

Killing Potentiation

This screen involves a two-step process. The advantage is that it monitors killing directly, rather than autolysis. Lysis is less pronounced in case of other bacteriocidal antibiotics such as quinolones or aminoglycosides as compared to cell wall inhibitors.

The first step is similar to the format used for MIC measurements. A low cell concentration (105/ml) is used in the presence of a sub-inhibitory concentration of a cidal antibiotic + test substances from the compound library. After an overnight incubation, a 2 µl aliquot is transferred to a well with 200 µl of fresh medium. After an 8 hour incubation, the plates are read, and the OD measurements will show high OD from wells where there was no growth inhibition, low OD from wells with complete growth inhibition, and undetectable OD from wells with substantial cell killing. The latter will indicate primary hit compounds. Those that have no effect on their own are putative anti-tolerance substances.

Growth Inhibition Potentiation

There are small differences in sensitivity of the sar strain and the wild type to growth inhibition by cidal antibiotics that can be measured and exploited in screening. A cidal antibiotic is added at a high enough concentration to produce 50-80% growth inhibition, and the cells are dispensed at low concentration in the MIC measurement format. Lack of growth in the presence of additional compounds from the library will signify hits. These are tested further and those that have no effect on growth inhibition on their own, but produce killing in the presence of sub-inhibitory antibiotic are likely anti-tolerance substances.

Identifying Genes for Screen Validation and Targeted Discovery of Anti-Tolerance Compositions

A number of genes that affect tolerance/persistence in several species have been elucidated. However, in no case have a large number of strains mutant in such tolerance genes been tested against a broad panel of antimicrobials. Our results indicate that many of these persistence/tolerance genes code for factors that affect cell survival under diverse lethal conditions such as under treatment with various antibiotic therapeutics. The following are optimal targets for drug discovery aimed at disabling tolerance and potentiating cidal antibiotics.

5.5. Other Targets

Lon Mutants in the Lon protease are sensitive to killing by quinolones. Lon homologs are broadly distributed among gram negative and gram positive species. An anti-Lon substance could eliminate tolerance to quinolone antibiotics.

SarA Mutants in this gene have been reported to be more sensitive to killing by cell wall inhibitors. According to our findings, sar mutants are more sensitive to other unrelated factors, including mitomycin. Importantly, mitomycin completely eradicated a population of S. aureus sar cells, but not the wild type. Anti-sar inhibitors could be used in combination with antibiotics to eliminate S. aureus infection, both conventional and biofilm. Sar is limited to some gram-positive bacteria.

Cell proteins that induce killing These targets can be used to screen for activating substances.

VncS Mutants in vncS of S. pneumoniae are resistant to killing by a broad range of bacteriocidal antibiotics. VncS may directly activate autolysis. VncS-targeting drugs are best utilized in inhibiting growth of gram positive bacterial species as vncS may be specific to this group.

Autolysins These come in a large variety of forms, but some of them have been specifically implicated in lyzing cells in response to antibiotics. LytA is present in S. pneumoniae; and LytM in S. aureus.

AmiB, MltB, SLT in E. coli These three have good homologs in P. aeruginosa.

Targets for increasing overall antibiotic susceptibility of biofilms According to our data, mutants in the major MexAB-OprM multidrug pump of P. aeruginosa produce biofilms that are considerably more susceptible to antibiotics, such as ofloxacin and tetracycline. A MexAB-OprM inhibitor will potentiate the antimicrobial action of antibiotics against biofilms.

Identifying Additional Targets

Apart from this short list based on what is known, it makes sense to select/screen for additional targets. Selection is straightforward - a Tn insertion library of cells is treated with cidal concentrations of antibiotic, cells are plated, and survivors are likely to have disruptions in genes activating killing. The clones that have higher MBC and normal MIC are those affected in tolerance. This selection approach will only produce targets that activate killing, thus drug discovery based on these targets will have to produce activator substances.

Screening an ordered Tn insertion library may be a more attractive approach. The simplest screen would be in the presence of ampicillin at a concentration that causes partial lysis of the wild type and can be done in microtiter plates. Mutants that have increased resistance to lysis will be affected in genes activating killing, while mutants with increased lysis will be in

genes coding for inhibitors of autolysis. The candidate mutants will be tested, and those that show wild type MICs and substantially changed MBCs are those affected in tolerance.

Validation of targets and hit compounds In the case of biofilms, for example, it is expected that a mutant of P. aeruginosa or S. aureus carrying a null mutation in a gene inhibiting tolerance (such as sar) will produce biofilms that will be highly susceptible to killing by a cidal antibiotic, unlike the wild type biofilm. Similarly, a hit compound is expected to potentiate the action of a cidal antibiotic against a biofilm made of wild type cells.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A method of inhibiting the survival of a persistent/tolerant microbial cell population comprising:

contacting the microbial population with a first composition which includes an antibiotic agent,

contacting the microbial biofilm with a second composition which includes an inhibitor of a microbial tolerance gene,

thereby inhibiting the survival of said microbial biofilm.

2. The method of claim 1, wherein the microbial cell population comprises a bacteria selected from the group consisting of: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Gardenerela vaginalis*, *Helicoberi pylori*, *Burkholderia psuedomalei*, *Haemophilus influenzae*.

3 The method of claim 1, wherein the antibiotic agent is selected from the group consisting of: penicillin, ampicillin, vancomycin, ciprofloxacin, tobramycin and mitomycin.

4. The method of claim 1, wherein the microbial tolerance gene is selected from the group consisting of: *sarA*, *relA*, *lon*.

5. The method of claim 1, wherein the persistent/tolerant microbial cell population comprises a microbial biofilm.

6. The method of claim 1, further comprising contacting the microbial cell population with a third composition which includes an inhibitor of a second microbial tolerance gene.

7. The method of claim 1, further comprising contacting the microbial cell population with a third composition which includes an activator of a microbial intolerance gene.

8. A method of inhibiting the survival of a persistent/tolerant microbial cell population comprising:

contacting the microbial population with a first composition which includes an antibiotic agent,

contacting the microbial biofilm with a second composition which includes an activator of a microbial intolerance gene,

thereby inhibiting the survival of said microbial biofilm.

9. The method of claim 8, wherein the microbial cell population comprises a bacteria selected from the group consisting of: *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa, Gardenerela vaginalis, Helicoberi pylori, Burkholderia psuedomalei, Haemophilus influenzae.*

10. The method of claim 8, wherein the antibiotic agent is selected from the group consisting of: penicillin, ampicillin, vancomycin, ciprofloxacin, tobramycin and mitomycin.

11. The method of claim 8, wherein the microbial intolerance gene is selected from the group consisting of: *SulA, lyt A, lytM, amiB, mltB and slt.*

12. The method of claim 8, wherein the persistent/tolerant microbial cell population comprises a microbial biofilm.

13. The method of claim 8, further comprising contacting the microbial cell population with a third composition which includes an inhibitor of a microbial tolerance gene.

14. The method of claim 8, further comprising contacting the microbial cell population with a third composition which includes an activator of a second microbial intolerance gene.

15. A composition for inhibiting the survival of a persistent/tolerant microbial cell population comprising a first composition which includes an an antibiotic agent, and a second composition which includes inhibitor of a microbial tolerance gene.

16. The composition of claim 15, wherein the microbial tolerance gene inhibitor is selected from the group consisting of: an antisense oliginucleotide and a ligand antagonist.

17. The composition of claim 15, wherein the antibiotic agent is selected from the group consisting of: penicillin, ampicillin, vancomycin, ciprofloxacin, tobramycin and mitomycin.

18. The composition of claim 15, further comprising a third composition which inhibits a second microbial tolerance gene target.

19. The composition of claim 15, further comprising a third composition which activates a microbial intolerance gene.

20. The composition of claim 15, wherein the microbial tolerance gene is selected from the group consisting of: *sarA*, *relA*, *lon*.

21. A composition for inhibiting the survival of a persistent/tolerant microbial cell population comprising a first composition which includes an an antibiotic agent, and a second composition which includes activator of a microbial intolerance gene.

22. The composition of claim 21, wherein the microbial intolerance gene activator is selected from the group consisting of: a ligand agonist, and a microbial intolerance gene delivery system.

23. The composition of claim 21, wherein the antibiotic agent is selected from the group consisting of: penicillin, ampicillin, vancomycin, ciprofloxacin, tobramycin and mitomycin.

24. The composition of claim 21, further comprising a third composition which inhibits a microbial tolerance gene target.

25. The composition of claim 21, further comprising a third composition which activates a second microbial intolerance gene.

26. The composition of claim 21, wherein the microbial tolerance gene is selected from the group consisting of: *SulA, lyt A, lytM, amiB, mltB and slt.*

27. A method for identifying a microbial tolerance gene target for treatment of a persistent/ tolerant microbial cell population comprising:

providing a library of mutated microorganisms comprising multiple independent clones, each having a tagged mutation in a gene, wherein the clones are all derived from a single wild-type strain of the microorganism;

contacting a mutated clone from said library with an antibiotic agent; and

comparing the survival of the clonal isolate having the tagged mutation with the survival of the wild-type strain under the same conditions;

wherein mutated clones having decreased survival in the presence of the antibiotic agent relative to the wild-type strain correspond to clones having a tagged mutation in a microbial tolerance gene, thereby identifying a microbial tolerance gene.

28. The method of claim 27, further comprising isolating the tagged mutated tolerance gene and identifying the protein expressed by the tolerance gene.

29. The method of claim 27 or 28, further comprising providing an antagonist of the microbial tolerance gene or protein.

30. The method of claim 29, wherein the antagonist is selected from the group comprising: an antisense oliginucleotide and a ligand antagonist.

31. The method of claim 27, whereint the antibiotic agent is selected from the group consisting of: penicillin, ampicillin, vancomycin, ciprofloxacin, tobramycin and mitomycin.

32. A method for identifying a microbial intolerance gene target for treatment of a persistent/ tolerant microbial cell population comprising:

providing a library of bacteria transformed with a bacterial gene overexpression vector library comprising a representative number of the genes from the bacterial microorganism functionally linked to a strong promoter;

contacting a single clone from said library of bacteria with an antibiotic agent; and

comparing the survival of the clonal isolate having a bacterial gene overexpression vector construct with the survival of an untransformed bacteria of the same strain and under the same conditions;

wherein a clonal isolate having decreased survival in the presence of the antibiotic agent relative to the untransformed bacteria of the same strain corresponds to a having a clone transformed with a bacterial intolerance gene, thereby identifying a microbial intolerance gene.

33. The method of claim 32, further comprising isolating the microbial intolerance gene and identifying the protein expressed by the microbial intolerance gene.

34. The method of claim 32 or 33, further comprising providing an agonist of the microbial intolerance gene or protein.

35. The method of claim 34, wherein the agonist is selected from the group comprising: a ligand agonist, and a microbial intolerance gene delivery system.

36. The method of claim 32, whereint the antibiotic agent is selected from the group consisting of: penicillin, ampicillin, vancomycin, ciprofloxacin, tobramycin and mitomycin.

37. A method of treating a bacterial infection with an antibiotic agent/ anti-microbial tolerance gene agent combination therapy comprising:

identifying at least one tolerance gene target for the bacteria by the method of claim 26;
contacting the bacterial infection with an antagonist of the microbial tolerance gene; and
contacting the bacterial infection with the antibiotic agent used to identify the microbial tolerance gene,

thereby treating the bacterial infection.

38. A method of treating a bacterial infection with an antibiotic agent and a microbial intolerance gene activating agent combination therapy comprising:

identifying at least one intolerance gene target for the bacteria by the method of claim 31;
contacting the bacterial infection with an agonist of the microbial intolerance gene; and
contacting the bacterial infection with the antibiotic agent used to identify the microbial tolerance gene,

thereby treating the bacterial infection.

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Published:
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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS AND COMPOSITIONS FOR POTENTIATING ANTIBIOTIC ACTION AGAINST PERSISTENT/TOLERANT PATHOGENIC MICROORGANISMS

(57) Abstract: The invention provides methods and reagents for the treatment of a microbial infection or decontamination of a microbial surface contamination, particularly those characterized by a population of persistent or tolerant cells such as occur in biofilm and certain planktonic bacterial populations. The invention is further directed to methods of identifying microbial tolerance gene targets and antagonsits of these microbial tolerance gene targets as well as for identifying microbial intolerance gene targets and agonists of these microbial intolerance gene targets.

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INTERNATIONAL SEARCH REPORT

Int ... nal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61P31/00 A61K48/00 A61K31/43

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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P,X	WO 01 12803 A (ELIOPOULOS GEORGE M ;MOELLERING ROBERT (US); GOLD HOWARD (US); INO) 22 February 2001 (2001-02-22) page 4, lines 25-30 page 8, line 5 - page 9, line 8 page 17, line 30 - page 18, line 4; claims 1,2,13	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 2003	-4 11. 2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gonzalez Ramon, N.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/26516

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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E	WO 02 085295 A (GREENBERG E PETER ;UNIV IOWA RES FOUND (US); BANGERA M GITA (US);) 31 October 2002 (2002-10-31) page 6, line 15 - page 7, line 10 page 12, line 26 - page 13, line 5; claims 2,3,6,14,24; examples 1-3; tables 1,2	1-3,5
Y	WO 99 13893 A (NIELSEN PETER E ;GOOD LIAM (SE); ISIS PHARMACEUTICALS INC (US)) 25 March 1999 (1999-03-25) abstract; claims 4,6	1-5, 15-17,20
Y	RODIONOV D. ET AL: "Direct correlation between overproduction of guanosine 3',5' bispyrophosphate (ppGpp) and penicillin tolerance in E. colli" J.BACTERIOLOGY, vol. 177, no. 15, August 1995 (1995-08), pages 4224-4229, XP001127469 see materials and methodsfigure 2	1-5, 15-17,20
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INTERNATIONAL SEARCH REPORT

Int nal Application No
PCT/US 01/26516

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NOVAK R ET AL: "PENICILLIN TOLERANCE GENES OF STREPTOCOCCUS PNEUMONIAE: THE ABC- TYPE MANGANESE PERMEASE COMPLEX PSA" MOLECULAR MICROBIOLOGY, BLACKWELL SCIENTIFIC, OXFORD, GB, vol. 29, no. 5, September 1998 (1998-09), pages 1285-1296, XP000890106 ISSN: 0950-382X abstract --- LEWIS K: "Riddle of biofilm resistance" ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC, US, vol. 45, no. 4, April 2001 (2001-04), pages 999-1007, XP002200811 ISSN: 0066-4804 page 1004, column 2 ---	1-5, 15-17,20
T		1-5, 15-17,20

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/26516

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 37,38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: partially 1, 2, 5, 15, 16
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-5, 15-17, 20 (all partially)

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: partially 1, 2, 5, 15, 16

Present claims relate to compounds/methods defined by reference to desirable characteristics or properties, namely "antibiotic" (claims 1,15)", "inhibitor of microbial tolerance gene" (claim 1), "antisense oligonucleotide" (claim 16), "ligand antagonist" (claim 16).

The claims cover all compounds/methods having these characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds/methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds/methods by reference to a result to be achieved.

Claims1, 2, 5, 15, 16 contain no technical feature describing the agents. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search for the first invention has been carried out for those parts of the claims which do appear to be clear, supported and disclosed, namely those compounds/methods recited in the examples and those mentioned in the claims 3, 4, 17, 20 with due regard to the general idea underlying the present application.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-5, 15-17, 20 partially

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic; b) contacting with a second composition that includes an inhibitor of a microbial tolerance gene of the antisense oligonucleotide type. Compositions containing the above.

---

2. claims: 1-5, 15-17, 20 partially

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic; b) contacting with a second composition that includes an inhibitor of a microbial tolerance gene of the ligand antagonist type. Compositions containing the above.

---

3. claims: 1-6,15-18, 20 partially

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic; b) contacting with a second composition that includes an inhibitor of a microbial tolerance gene of the antisense oligonucleotide type; c) contacting with a third composition that includes an inhibitor of a second microbial tolerance gene. Compositions containing the above.

---

4. claims: 1-6,15-18, 20 partially

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic; b) contacting with a second composition that includes an inhibitor of a microbial tolerance gene of the ligand antagonist type; c) contacting with a third composition that includes an inhibitor of a second microbial tolerance gene. Compositions containing the above.

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5. claims: 1-5, 7, 8,1 3, 15-17, 19, 21, 24, 26 partially

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic b) contacting with a second composition that includes an inhibitor of a microbial tolerance gene of the antisense oligonucleotide type; c) contacting with a third composition that includes an activator of a microbial intolerance gene. Compositions containing the above.

---

6. claims: 1-5, 7, 8, 13, 15-17, 19, 21, 24, 26 partially

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic b) contacting with a second composition that includes an inhibitor of a microbial tolerance gene of the ligand antagonist type c) contacting with a third composition that includes an activator of a microbial intolerance gene. Compositions containing the above.

---

7. claims: 8-12, 21-23 partially

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic; b) contacting with a second composition that includes an activator of a microbial intolerance gene of the ligand agonist type. Compositions containing the above.

---

8. claims: 8-12, 21-23 partially

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic b) contacting with a second composition that includes an activator of a microbial intolerance gene of the microbial intolerance gene delivery system type. Compositions containing the above.

---

9. claims: 8-12, 14, 21-23, 25 partially

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic; b) contacting with a second composition that includes an activator of a microbial intolerance gene of the ligand agonist type; c) contacting with a third composition that includes an activator of a second microbial intolerance gene. Compositions containing the above.

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10. claims: 8-12, 14, 21-23, 25 partially

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Method of inhibiting survival of persistent/tolerant microbial population comprising: a) contacting with a first composition including an antibiotic; b) contacting with a second composition that includes an activator of a microbial intolerance gene of the microbial intolerance gene delivery system type; c) contacting with a third composition that includes an activator of a second microbial tolerance gene. Compositions containing the above.

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11. claims: 27-31, 37

Method for identifying a microbial tolerance gene. Use of the same for treating bacterial infection.

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12. claims: 32-36, 38

Method for identifying a microbial intolerance gene. Use of the same for treating bacterial infection.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ınal Application No
PCT/US 01/26516

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